NEURONAL NETWORK ANALYSES IN VITRO OF ACUTE INDIVIDUAL AND COMBINED RESPONSES TO FLUOXETINE AND ETHANOL

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Thesis Prepared for the Degree of

MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS

August 2002

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Xia,Yun. Neuronal Network Analyses *in vitro* of Acute Individual and Combined Responses to Fluoxetine and Ethanol. Master of Science (Biology), August 2002, 65 pp., 5 tables, 29 figures, references, 76 titles.

Embryonic murine neuronal networks cultured on microelectrode arrays were used to quantify acute electrophysiological effects of fluoxetine and ethanol. Spontaneously active frontal cortex cultures showed highly repeatable, dose-dependent sensitivities to both compounds. Cultures began to respond to fluoxetine at 3 μM and were shut off at 10-16 μM. EC50s mean ± S.D. for spike and burst rates were 4.1 ± 1.5 μM and 4.5 ± 1.1 μM (*n*=14). The fluoxetine inhibition was reversible and without effect on action potential wave shapes. Ethanol showed initial inhibition at 20 mM, with spike and burst rate EC50s at 52.0 ± 17.4 mM and 56.0 ± 17.0 mM (*n*=15). Ethanol concentrations above 100 -140 mM led to cessation of activity. Although ethanol did not change the shape and amplitude of action potentials, unit specific effects were found. The combined application of ethanol and fluoxetine was additive. Ethanol did not potentiate the effect of fluoxetine.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Guenter W. Gross for his guidance and support. I thank my committee members, Dr. Thomas L. Beitinger and Dr. Jannon L. Fuchs for their help and inspiration. I appreciate all of the help from the research group, especially the cell culture and multielectrode array fabrication staff that consistently supported my research efforts.

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CHAPTER 1

INTRODUCTION

1.1 Overview of Cultured Neuronal Networks

It has been suggested that cultured neuronal networks can be used as experimental platforms for the study of neurotoxicology and drug development (Gross et al., 1997b). The cultured neural networks display stable spontaneous and long-term activity that can be maintained for several months (Gross and Kowalski, 1991). With high reproducibility and sensitivity to neuroactive chemicals, such networks can sense the changes of the *in vitro* environment and test for the following effects: (1) changes in metabolism, (2) influence on synaptic mechanisms, (3) alterations in membrane permeability, and (4) changes in other mechanisms responsible for spike generation and formation of spike patterns (Gross et al., 1997b).

The Center for Network Neuroscience (CNNS) at the University of North Texas (UNT) was the first entity to demonstrate that neuronal networks, grown on arrays of microelectrodes *in vitro*, were uniquely suited for use as broadband biosensors (Gross et al., 1992, 1995, 1997a, b). Based on pharmacological data, it is apparent that all receptors, synapses, and cellular mechanisms responsible for pattern generation in a specific CNS tissue are retained and represented in culture (Gramowski et al., 2000; Morefield et al., 2000; Keefer et al., 2001 a, b, c). Consequently, I hypothesize that any compound that alters these mechanisms to a level that affects the performance and life support of an animal should be reflected in changes in the spontaneous activity of the

networks. These changes are considered “cell culture correlate responses” to the altered behavioral or life-threatening responses that occur in animals. Therefore, the networks are physiological sensors as they appear to be capable of responding to any compounds, in approximately the same concentration range, that could destroy or alter the functions of an intact mammalian nervous system. To validate neuronal network responses in culture, two common and frequently used compounds (fluoxetine and ethanol) have been investigated and their effects and potency have been compared to *in vivo* studies.

1.2. Fluoxetine and the Possible Mechanisms Underlying Its Effects

Fluoxetine has several trade names, such as Fontex, Prozac, Fluoxetine ratiopharm, Fluoxetine selena, Seroscand. It is used for treating depression, obsessive-compulsive disorders, bulimia nervosa, and panic/agoraphobia syndrome.

Drugs effective in treating depression mainly act on serotonergic and noradrenergic systems. The major fibers of the serotonergic system are in the raphé nuclei of the brainstem. The cells from more caudal nuclei innervate the spinal cord and this pathway is involved in response to painful stimuli. The cells from the more rostral nuclei project diffusely throughout the brain and this pathway is involved in modulation of the sleep-waking cycle and mood. Serotonin deficit can be caused by several things: not enough production; not enough receptor sites; unusually rapid degradation or reuptake. Serotonin deficiency is correlated with depression (Asberg et al., 1976). There are several key steps in serotonin transmission. Vesicles of serotonin molecules are released from the axonal

boutons of the presynaptic cells into the synaptic gap. These molecules then bind to serotonin receptors on the postsynaptic nerve cell membrane and pass along their chemical message. The actions of serotonin are terminated by reuptake into the presynaptic cells. Fluoxetine is a selective inhibitor of this uptake and thus increases the concentration of serotonin in synaptic clefts. The effect of increased serotonin concentration includes increase in firing or cessation in firing of postsynaptic cells, depending on the receptor types activated (Andrade, 1998).

With long-term efficacy, acceptable side effects, and easy administration, fluoxetine has been marketed in more than 90 countries and is used by more than 40 million people worldwide. In addition to being a useful antidepressant, fluoxetine has become a tool to study the mechanisms of depression (Delgado et al., 1990) and other psychiatric disorders (Hudson and Pope, 1990). In order to use this drug more effectively, extensive studies are still needed to find the involvement of serotonergic pathway in depression and the secondary effects of this drug on central nervous system.

The binding of [^{3}H]-fluoxetine is used to investigate 5-HT uptake in brain tissue (Wong et al., 1993). Saturable binding in cerebral cortical membranes occurs in the concentration range of 0.5-10 nM of [^{3}H]-fluoxetine. Voltage-clamp recordings using whole-cell configuration of the patch clamp technique indicates that fluoxetine inhibits voltage-activated K^{+}, Ca^{2+}, and Na^{+} currents in pheochromocytoma cells (Hahn et al., 1999). Mukherjee (1998) used radiolabeled ^{18}F-fluoxetine as a radiotracer of fluoxetine and showed that ^{18}F-fluoxetine binding to rat brain was mainly subcellular and the total

binding in all main areas was uniform. Fluoxetine has high selectivity for the 5-HT transporter (Wong et al., 1995) and low affinity for neurotransmitter receptors. Thus, it has few side effects at low concentrations.

Increasing evidence suggests that the serotonergic system can modulate seizures and that fluoxetine may have an anticonvulsant action in animal models. This anticonvulsant action is due to an increase of endogenous 5-HT transmission. Fluoxetine (3.5 nmol) protected rats from limbic motor seizures evoked focally from area tempestas, an epileptogenic site in deep rostral piriform cortex. The 5-HT antagonist partially reverses the anticonvulsant action of intranigral fluoxetine, and depletion of endogenous 5-HT completely abolishes the anticonvulsant action of intranigral fluoxetine. These observations suggest that the antiseizure action of fluoxetine in substantia nigra is due to an enhancement of synaptic action of 5-HT on multiple 5-HT receptors. Endogenous 5-HT transmission in substantia nigra therefore is capable of limiting the development and propagation of seizure activity generated in limbic circuits (Pasini et al., 1996).

In my research, spontaneously active monolayer neuronal networks cultured on microelectrode arrays were used to determine the effect of fluoxetine on cultured embryonic murine frontal cortex. The major observations included suprathreshold electrophysiological changes, reversibility, and irreversibility at different concentrations. This research further supports the efforts of this laboratory to use neuronal networks as screening platforms for neuropharmacological and neurotoxicological studies. The data

obtained also support the earlier suggestions that such networks can be used as tissue-based biosensors.

1.3 Overview of Ethanol

Ethanol (CH_3CH_2OH, ethyl alcohol) belongs to a group of chemical compounds that contain a hydroxyl group (–OH) bonded to a carbon atom. The primary target of ethanol is the central nervous system. In human, acute ethanol intoxication causes impaired judgment, lower self-control, inappropriate behavior, slurred speech, incoordination, and unsteady gait. These changes reflect the influence of ethanol on the brain, especially prefrontal area, temporal area and cerebellum. In general, functional changes are dose-related. Ethanol intoxication is usually defined as a blood alcohol level (BAL) of 0.1 g/dL (22 mM). A BAL of 0.05-0.10 g/dL (11-22 mM) causes slight impairment of balance, speech, reduced judgment and reasoning. A BAL above 0.5 g/dL (110 mM) leads to coma and death. Chronic ethanol abuse produces tolerance, dependence, withdrawal, anxiety, and hyperexcitability.

Although ethanol is a familiar compound, its molecular and cellular mechanisms are still not well understood. The lipid theory of alcohol action was advanced for many years (Meyer, 1937; Mullins, 1954). It proposed that the disturbance of membrane lipids by alcohol influenced the function of membrane proteins which were involved in modulation of neuronal activity. However, much criticism has been directed at this theory based on the observation that alcohol did not exert direct effects on lipid bilayer membranes. The

studies of neuronal firing revealed that ethanol could influence the spontaneous spike firing in the central nervous system. It was reported that the intravenous administration of ethanol to rats reduced the firing rates of inferior olivary neurons (Harris and Sinclair, 1984). Siggins et al. (1987) found that, of spontaneously firing CA1 pyramidal neurons, 10-350 mM ethanol decreased the firing rates in 50%, had no effect in 29%, generated biphasic effect in 12%, and increased firing in 9%. They also observed that the application of 50-200 mM ethanol in hippocampal slices increased firing in 32% of the neurons, decreased firing in 24%, had biphasic effect in 32%, and generated no obvious effect in 12%. The *in vitro* study of hippocampal pyramidal cells showed that ethanol caused excitatory, inhibitory, biphasic effect, or had no effect on neuronal discharge. The effect of ethanol on noradrenergic neurons in locus coeruleus was studied by Pohorecky and Brick (1977). It was found that intraperitoneal administration of ethanol decreased the spontaneous firing in 62%, increased in 22%, and had no effect in 16% of the cells tested. There were also studies on the cellular mechanisms of ethanol effect, such as voltage-gated ion channels and ligand-gated ion channels (Aguayo, 1990; Benson et al., 1989; White et al., 1990).

Although many studies have been done on ethanol, the knowledge of the effect of ethanol on different brain regions is incomplete (Little, 1999). In this research, the response of cultured frontal cortex networks to ethanol has been studied, which sheds light on the action of ethanol on frontal cortex.

1.4 Objectives of Study

The major purpose of this research was to investigate the responses of cultured frontal cortex networks to fluoxetine and ethanol and to compare these responses to the results obtained in other investigations, especially *in vivo* studies. This allows a comparison of *in vitro* dose-response data with responses of organisms and helps substantiate the use of cultured networks as pharmacological assay systems and biosensors.

Specific aims were to:

(1) Characterize the electrophysiological effects of fluoxetine on murine embryo frontal cortex cultures.

(2) Examine the electrophysiological effects of ethanol on murine embryo frontal cortex cultures.

(3) Determine the dose-response relationships of fluoxetine and ethanol.

(4) Compare the effects to those obtained in brain slices and *in vivo* studies.

(5) Determine if both substances together generate additive effects.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

Dissociated embryonic murine frontal cortical tissues were cultured following Ransom (Ransom et al., 1977) with some modification that included the use of DNase to dissociate tissue (Gross, 1979; Gross and Kowalski, 1991).

To ensure cell adhesion to the insulation material, butane flaming was used to oxidize the methyl group of polysiloxane to hydroxyl groups and form a hydrophilic surface (Lucas et al., 1986). Flaming through a stainless steel mask produced a central adhesion island (3 mm in diameter) centered on a 0.8 × 0.8 mm recording matrix and a separate medium conditioning area. Poly-D-lysine and laminin, used for substrate preparation, adhered only to the flamed regions. Cortical tissues were obtained from 15-16 day old Balb-C/ICR murine embryos. The tissues were dissociated mechanically (mincing and triturating) and enzymatically (with DNase). Figure 1 shows the procedures of the cell culture performed by the CNNS culture staff.

The tissue were seeded onto the prepared areas of the multielectrode arrays (MEAs) and maintained in Dulbecco's modified minimal essential medium (DMEM), supplemented with 5% horse serum and 5% fetal bovine serum. Approximately 8×10^5 cells (neurons and glia) in 1 ml aliquot were seeded on each MEA with medium confined to a 4 cm^2 area by a silicone gasket (Gross and Kowalski, 1991; Gross and Schwalm,

1994). Cells were maintained at 37°C in an atmosphere with 10% CO_2 and 90% air for a week. Thereafter, cells were fed biweekly with DMEM containing 5% horse serum.

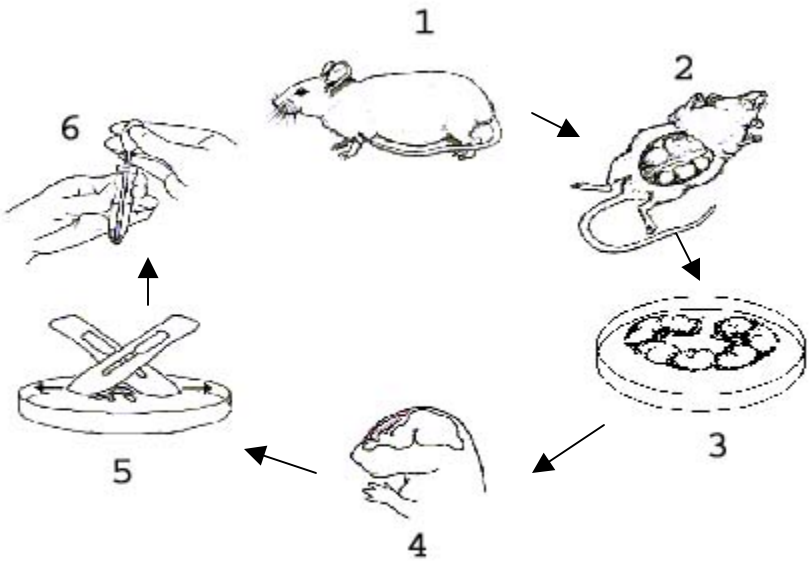


Figure 1. Major procedures of cell culture (Drawn by Emese Dian)
1. Balb-C/ICR mice are mated for 24 hours, fifteen to sixteen days before culturing.
2. A single pregnant mouse is anesthetized for each batch of cultures, sacrificed by cervical dislocation, and dissected under sterile conditions to remove the uterus.
3. Ten to fourteen embryos are delivered from the uterus and placed in D1SGH medium.
4. Each fetus is decapitated and has its frontal cortex removed.
5. Tissue is minced with two sterile scalpel blades.
6. Minced cortex is triturated in 5 ml DMEM, 5% horse serum, 5% fetal bovine serum, 2% B27, 8 µg vitamin C/ml (DMEM5/5).

The central island of the MMEP culture overlaid the recording matrix and typically grew into a confluent glial carpet. Neuronal somata generally developed on the top of carpet, and axonal processes grew both below and above of glial layer. The cultures displayed spontaneous activity in the pattern of random spiking at about 5-7 days. They

were considered mature after 3 weeks and remained spontaneously active and pharmacologically responsive for more than half a year (Gross and Kowalski, 1991; Gross, 1994). Figure 2 shows a three-week-old culture on the 64-electrode MEA.

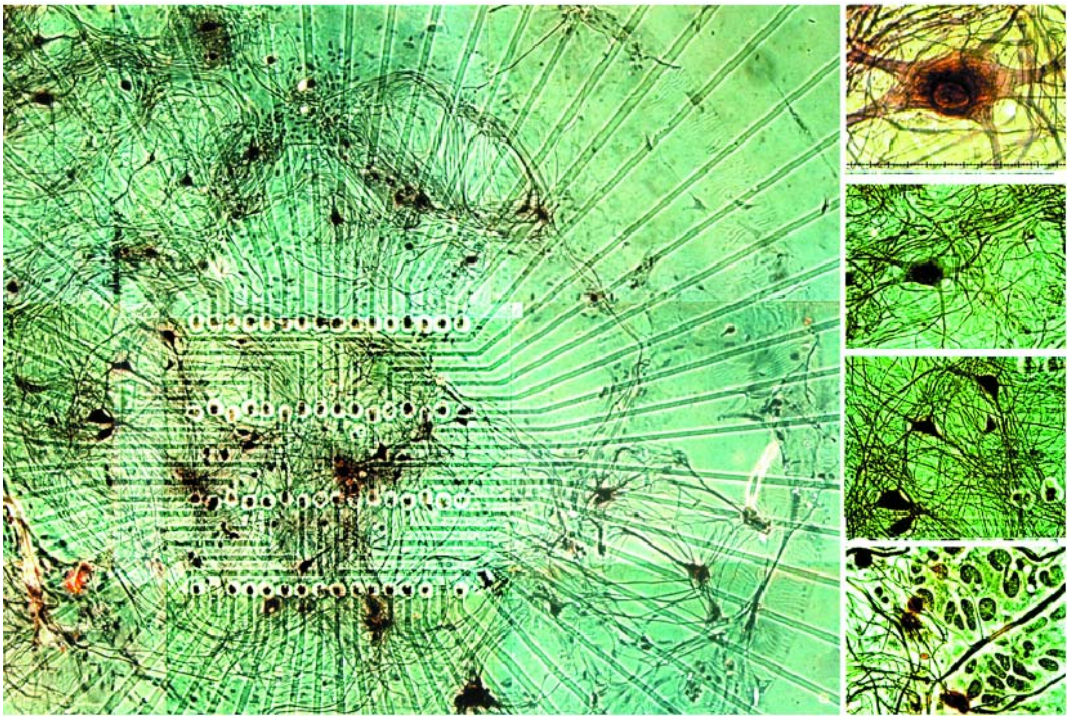


Figure 2. Fixed and Bodian stained network cultured on a 64-electrode recording matrix. Transparent indium-tin oxide conductors are 8 μm wide and terminate in 4 rows and 16 columns. Right panel shows morphology of multipolar neurons within the network (CNNS archives).

2.2 Preparation of Microelectrode Arrays

The techniques for microelectrode plate fabrication and preparation have been described before (Gross, 1979; Gross and Lucas, 1982; Gross et al., 1985; Gross and Kowalski, 1991; Gross, 1994). MEAs consisted of an array of photoetched electrodes.

The MEA (5×5 cm) was prepared from 1.2 mm thick indium-tin oxide (ITO) sputtered glass (soda line glass with a 100 nm quartz layer, Applied Films Corp., Boulder, CO), photoetched, spin-insulated with polysiloxane resin, cured at high temperature, and deinsulated at the electrode tips via a single laser pulse (Gross, 1979). The exposed ITO was electroplated with a thin layer of colloidal gold to decrease the interface impedance to 1-2 Mohm at 1K Hz (Gross et al., 1985).

2.3 Recording

Recording chambers fabricated by the CNNS were used to maintain neural networks in a constant bath of recording medium. This design allowed rapid medium changes and short-term pharmacological manipulation. The assembly consisted of an aluminum base holding the MEA and a stainless steel chamber. Zebra strips (carbon-filled silicone elastomere, Fujipoly, Cranford, NJ) were placed between the amplifier circuit board and the MEA to ensure electrical contact (Gross and Kowalski, 1991). Figure 3 depicts the assembly of an open recording chamber.

The extracellular spike activity was recorded with a computer-controlled, 64-channel multiamplifier system (Plexon Inc., Dallas, TX). Preamplifiers were placed on the microscope stage to both sides of the recording chamber and connected to the MEA. Total system gain was mostly set to 10 K with bandpass usually set at 500 Hz to 6 KHz. Activity was displayed on oscilloscopes (Tektronix Inc., Beaverton, Oregon). Spike rates from active channels were integrated and displayed on an 8-channel Soltec strip chart

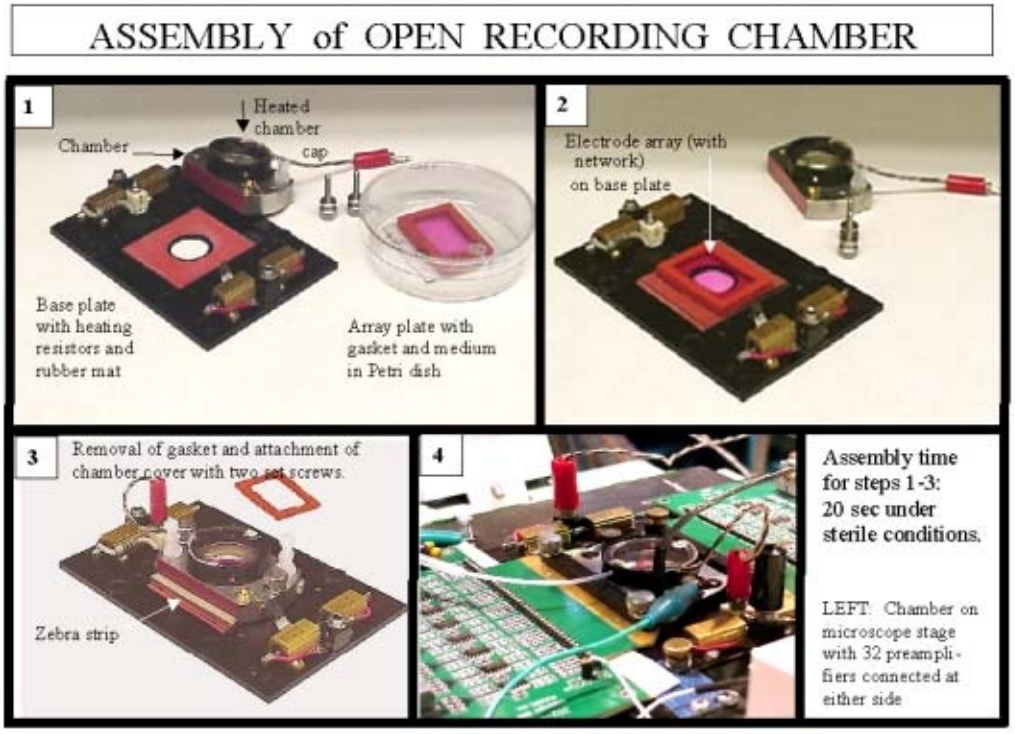


Figure 3. Assembled chamber on a microscope stage with 32 preamplifiers attaching each side. A gas line provides a humidified mixture of 10% CO_2 and 90% air. Four power resistors heat the chamber to 37^{o}C. The cap, containing a heated ITO cover, provides a clear window for light microscopy and confines the proper atmosphere. A stainless steel chamber confines the culture medium (CNNS archives).

recorder 8K40 (Soltec Corporation, San Fernando, CA) with rectification followed by analog integration with a resulting integration constant from 200 ms to 1 s. The amplifier ground was connected to the stainless steel chamber holding the culture medium.

Cultures used for experiments were at least 21 days *in vitro* (d.i.v.) and selected to show spontaneous activity. Assembled MEAs were placed on a microscope stage and

sustained at 37^{o}C. The pH was maintained at 7.4 with a continuous supply (15 ml/min) of humidified 10% CO_2 and 90% air confined under a special cap. After assembly, individual neuronal activity was monitored at each electrode in real time with analog amplifier to provide single unit spike information. Spikes were discriminated using the Plexon "box" templates. Action potential profiles passing through two "boxes" were identified as specific active units. Spikes with high signal-to-noise ratios were usually selected. Whole network data were analyzed with burst recognition and analysis programs.

Wash medium consisted of fresh DMEM and 5% horse serum. Usually, 60 minutes were allowed for the activity to stabilize before recording. Reference activity is the activity recorded after washing with fresh medium. During the course of experiment, the osmolarity and pH were monitored carefully to assure an optimal environment. Osmolarity was measured with a vapor pressure osmometer (Wescor Inc., Utah) at the beginning of each experiment and thereafter every three hours. The pH was monitored with an Accumet Combination Micro Electrode with Calmol reference (Fisher Scientific) using only 100 μl sample volumes.

2.4. Data Analyses

Real-time spike production was displayed on oscilloscopes. In view of large amount of data and ubiquitous feature of spike clusters (bursts), simplification of data acquisition and analysis was necessary. Spike integration was a convenient tool that revealed the

major modes of network activity via burst patterns. With integration, the integrated burst amplitude and duration could be accurately determined. This burst pattern analysis provides highly useful information without complex analysis of individual spike data (Gross, 1994).

Each electrode could monitor more than one unit. For electrodes having only one unit, integrated amplitude reported instantaneous spike frequency and area under the curve showed the total spike production. The integrated burst profiles were quantified with two thresholds. Low threshold (T1) identified the starting point of burst and high threshold (T2) determined sufficient size needed for burst analysis (Fig. 4).

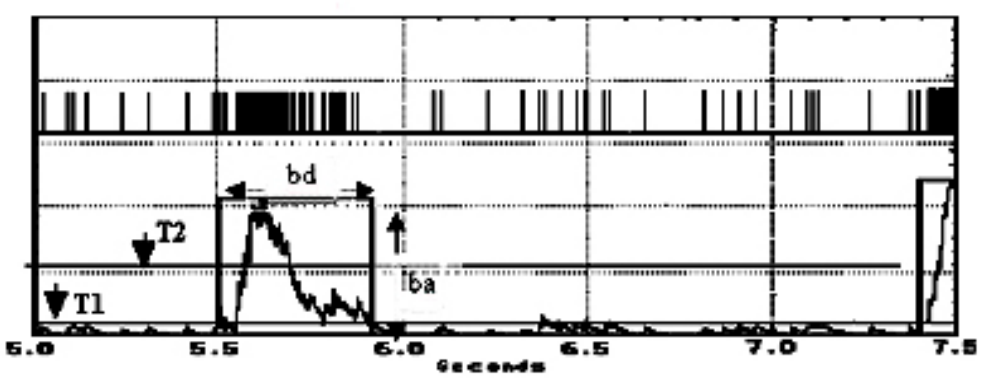


Figure 4. Integration of spike data. Burst establishment and quantification were based on two thresholds: T1 and T2. T1 determined the beginning and the end of a burst. T2 chose the burst for qualification. ba = burst amplitude; bd = burst duration.

To determine the changes of network activity with time, mean network spike rates and burst rates of all channels over the course of whole experiment were plotted. Plots

were also used to determine the initial effective concentration and shut-off concentration. Other data such as waveform, integrated burst amplitude and burst duration also were analyzed.

2.5 Pharmacological Manipulations

2.5.1 General Procedures

Drugs were micropipetted to the culture medium at 12, 3, 6 and 9 o'clock positions near the edge of the chamber to obtain an even distribution. To remove the test drug, syringes were used to extract the "old" medium and to add fresh wash medium.

The applied concentrations ranged from 0.5 to 75 μM for fluoxetine and from 10 to 180 mM for ethanol. Concentrations were increased sequentially and activity was recorded continuously during the experimental episode. Each concentration was maintained for at least 30 minutes. The wash medium was added to the chamber immediately after the removal of previous medium. Fluoxetine ($C_{17}H_{18}F_3NO$, HCl) and Ethanol (CH_3CH_2OH, 100%, 200 proof) were obtained from Sigma Aldrich, Inc. (St. Louis, MO; Milwaukee, WI).

2.5.2 Specific Manipulations for Ethanol Experiments

Ethanol is volatile and the CO_2 / air flow used to control pH accelerated the change of ethanol concentration in the chamber. Therefore, the maintenance and monitoring of ethanol concentrations were critical. Ethanol was added to the wash bottle at the same

concentration as that of the recording chamber (Fig. 5). Before administering ethanol to the recording chamber, the wash bottle with same concentration of ethanol was connected to the gas line that supplied CO_2 and air mixture. Concentrations of ethanol were determined with an alcohol diagnostic kit (Sigma Diagnostics Inc., St Louis, MO).

The major steps to determine alcohol concentration included:

1. Prepare alcohol reagent solution (ARS)
2. Blank: 3 mL ARS + 10 μL wash medium.
3. Test: 3mL ARS + 10 μL sample
4. Incubate
5. Measure absorbance at wavelength of 340 nm (A340)
6. Calculate: Alcohol (mmol/L) = A340 × 48.4

2.6 Statistical Analyses

The network mean spike rates and burst rates are binned data (bin size = 60 seconds) averaged across the network. Independent t tests were used to test the difference of two means. One-way ANOVA followed by Dunnett's multiple range test was used to test the difference among several means. $P < 0.05$ was defined as statistically significant.

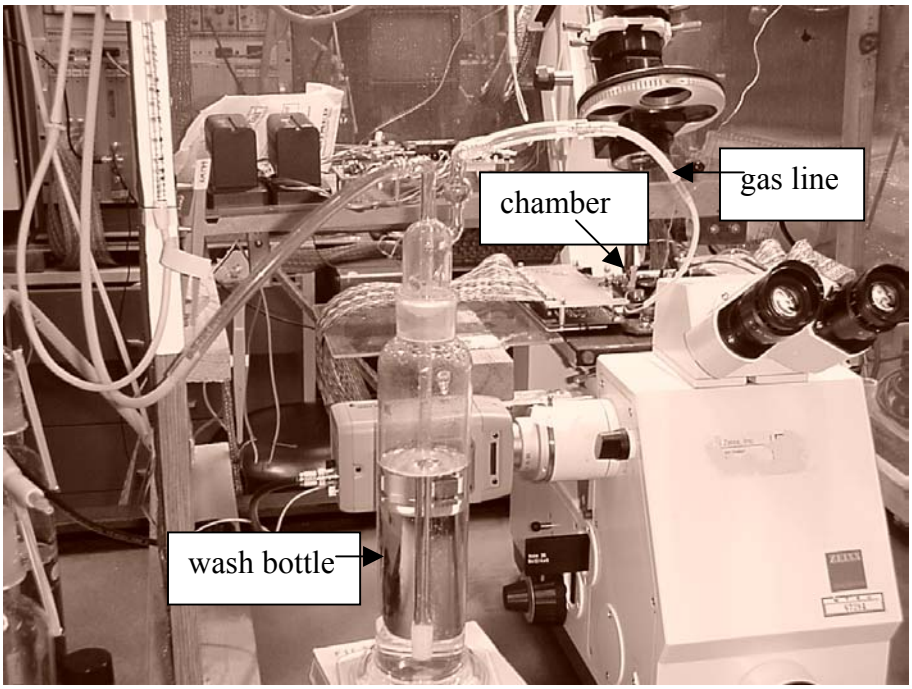


Figure 5. A Recording chamber on the microscope stage. The left side of 32 preamplifiers and associated cables can be seen. To maintain ethanol concentration, ethanol was added to the wash bottle at the same concentration as that in the recording chamber. The gas line provided a mixture of ethanol vapor, 10% CO_2, and 90% air.

CHAPTER 3

RESULTS

3.1 Normal Spontaneous Activity of Frontal Cortex Networks

The ages of tested frontal cortex cultures ranged from 21 to 61 days *in vitro.* Active units were selected with signal-to-noise ratios (SNR) equal to or greater than 2:1. The cultures tested had an average electrode yield of 34.4 % (20 channels) with a mean SNR of 3. Big signal amplitudes often reached 300 μV and maximum SNR reached 8:1. Figure 6 shows the real-time display of frontal cortex networks activity on a Plexon display window. The three panels from left to right show: activity of currently selected channel; 40-second raster plot of multichannel activity (n = 30); spike display of all selected units.

Figure 7 displays waveforms of native activity from two units after unit discrimination. The large unit measured 300 μV and small unit measured 150 μV. The multichannel recording allows real-time monitoring of network activity.

Frontal cortex cultures often display spontaneous activity with phasic and tonic spiking. Bursting is highly coordinated and ubiquitous over most selected channels. Usually, bursts occurred simultaneously over the discriminated units with some "random" spiking among several units between bursts. Mean spike and burst rates were averaged over the network using 60 seconds as bin. Generally, mean network spike rates fell into the range of 200-1200/minute and burst rates ranged from 10/minute to 50/minute. A typical raster plot of general firing pattern is shown in Figure 8. The 30-

second panel shows coordinated bursts and some interburst spiking among the 19 channels.

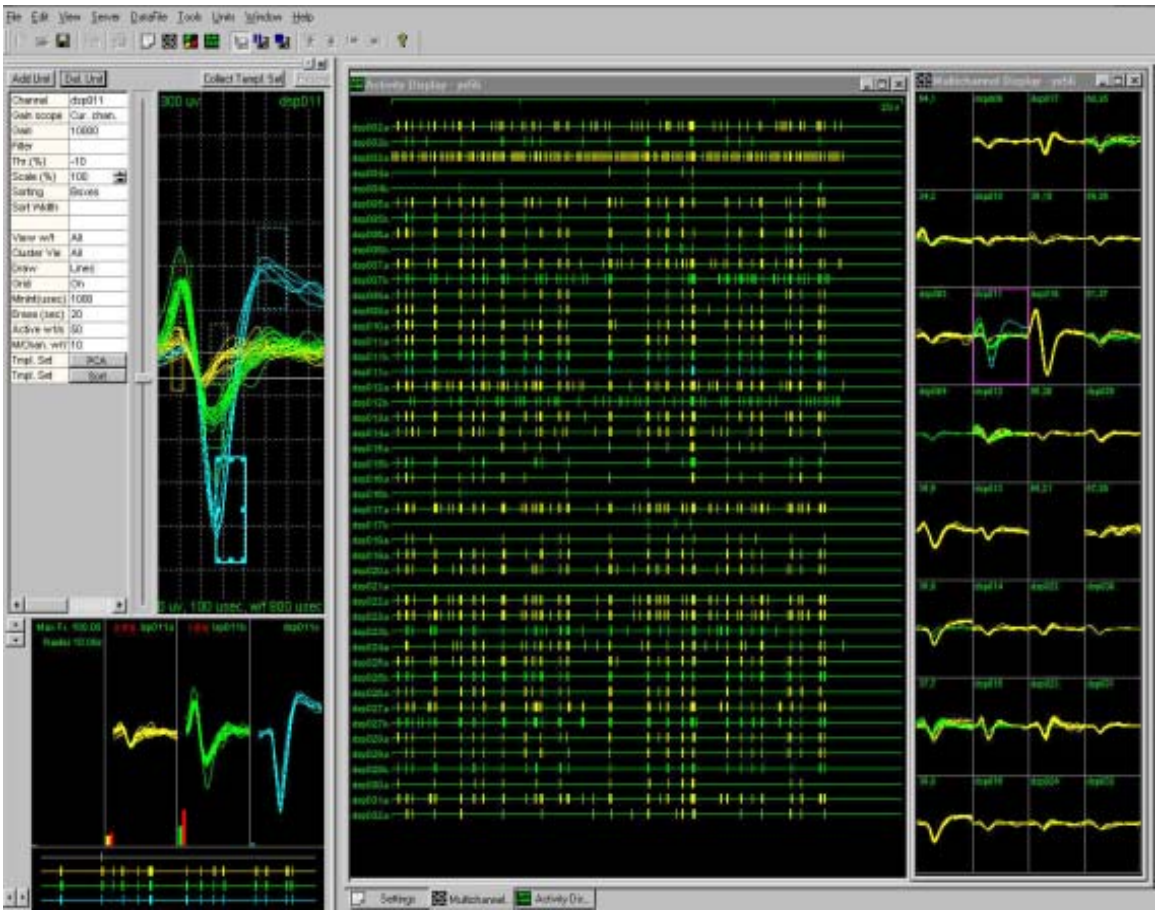


Figure 6. Display of real-time activity of frontal cortex networks. The three panels from left to right show: activity of current channel; 40-second raster of multichannel activity; spike display of selected units. Note that the current channel has three waveforms that could be discriminated. After discrimination, each unit is given its own raster channel.

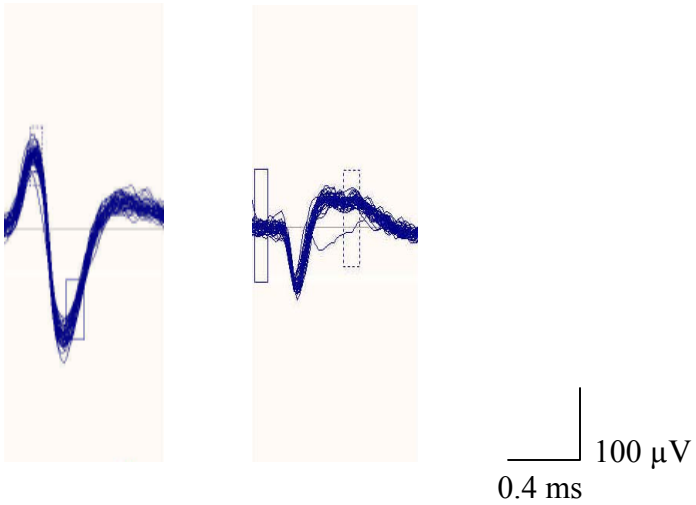


Figure 7. Two panels of waveforms recorded from two units after spike discrimination. The waveforms represent digitized action potentials.

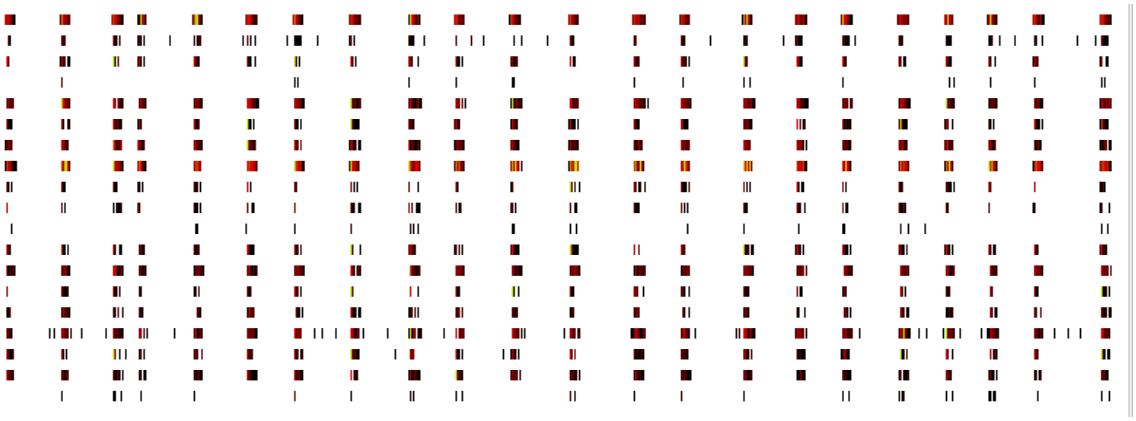


Figure 8. A raster plot of digitized activity for a 30-second episode. Coordinated activity can be seen among 19 channels in the episode. Single lines represent digitized action potentials for which time stamps are stored as .plx files.

3.2 Effects of Fluoxetine on Cultured Neuronal Networks

Fourteen frontal cortex cultures were used for the study of fluoxetine. The ages of the cultures ranged from 21 to 58 days *in vitro.* Table 1 lists the fluoxetine experiments.

Experiment	Date	Age (d.i.v)	% Active channels	Max SNR	Avg SNR
YX39	07/29/2001	58	34	4	2.5
YX43	08/31/2001	28	44	6	3
YX44	09/07/2001	28	44	5	2.5
YX45	09/14/2001	44	64	3	2.5
YX47	09/28/2001	34	56	8	2.7
YX48	10/04/2001	54	30	4	3
YX51	10/26/2001	29	25	5	3
YX53	11/09/2001	21	52	10	4
YX55	11/16/2001	28	32	3.5	2.5
YX56	12/12/2001	46	50	8	3.5
YX57	12/13/2001	47	13	6	3
YX58	01/14/2002	31	55	8	3
YX59	01/15/2002	26	20	6	3.5
YX60	02/04/2002	30	33	9	4
Mean		36	39	6	3

Table 1. Frontal cortex cultures used in fluoxetine study. d.i.v = days *in vitro*; SNR = signal-to-noise ratio.

No apparent change of waveform amplitude or shape was observed under exposure to fluoxetine. Figure 9 (A) shows the native waveforms and 9 (B) shows the waveforms when exposed to 6 µM fluoxetine. The waveforms did not change before activity was completely shut off.

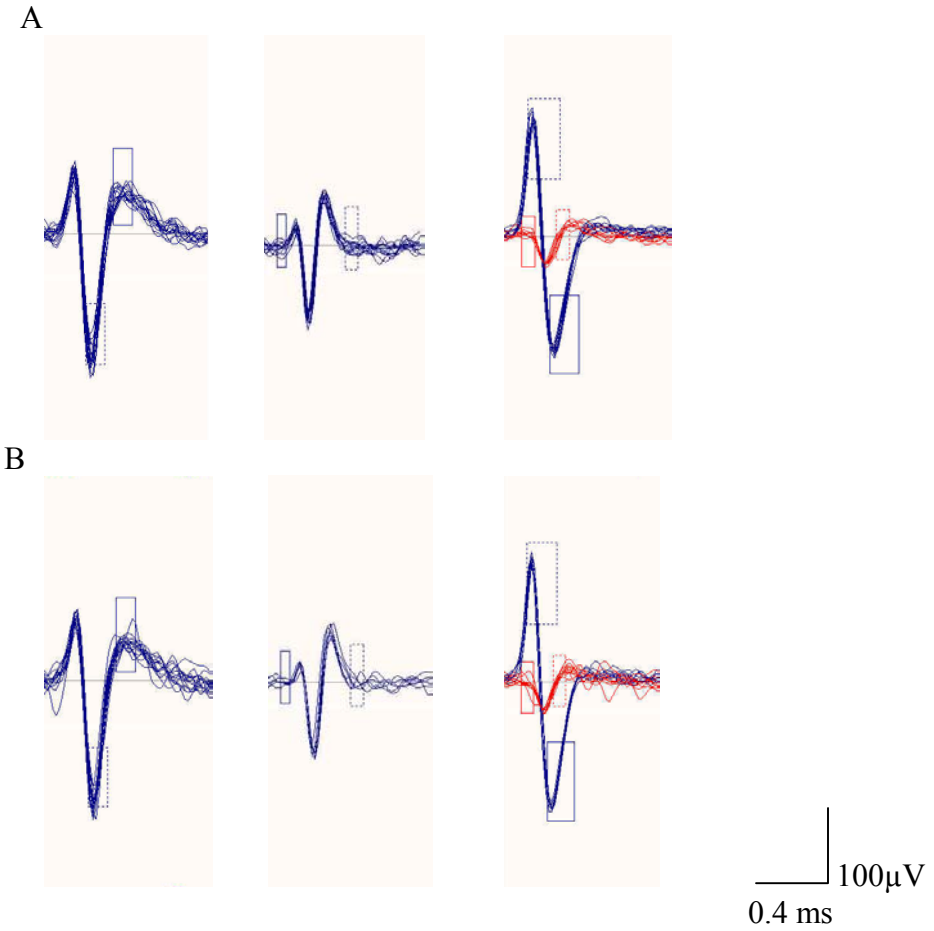


Figure 9. Panels of representative action potential waveforms from the same recorded units of frontal cortical culture (A) in native state and (B) under exposure to 6 µM fluoxetine for 30 minutes. Fluoxetine did not induce noticeable changes in the amplitude or shape of action potential.

It was observed that fluoxetine produced a uniform effect across the units. Figure 10 shows the rate histograms of 42 units. The horizontal axis represents time and the vertical axis represents spike rates. The rate histograms of all units display a diminution in response to 8 μM fluoxetine (vertical line). Although all units decreased their spike rates, the level activity plateaus after response stability were different.

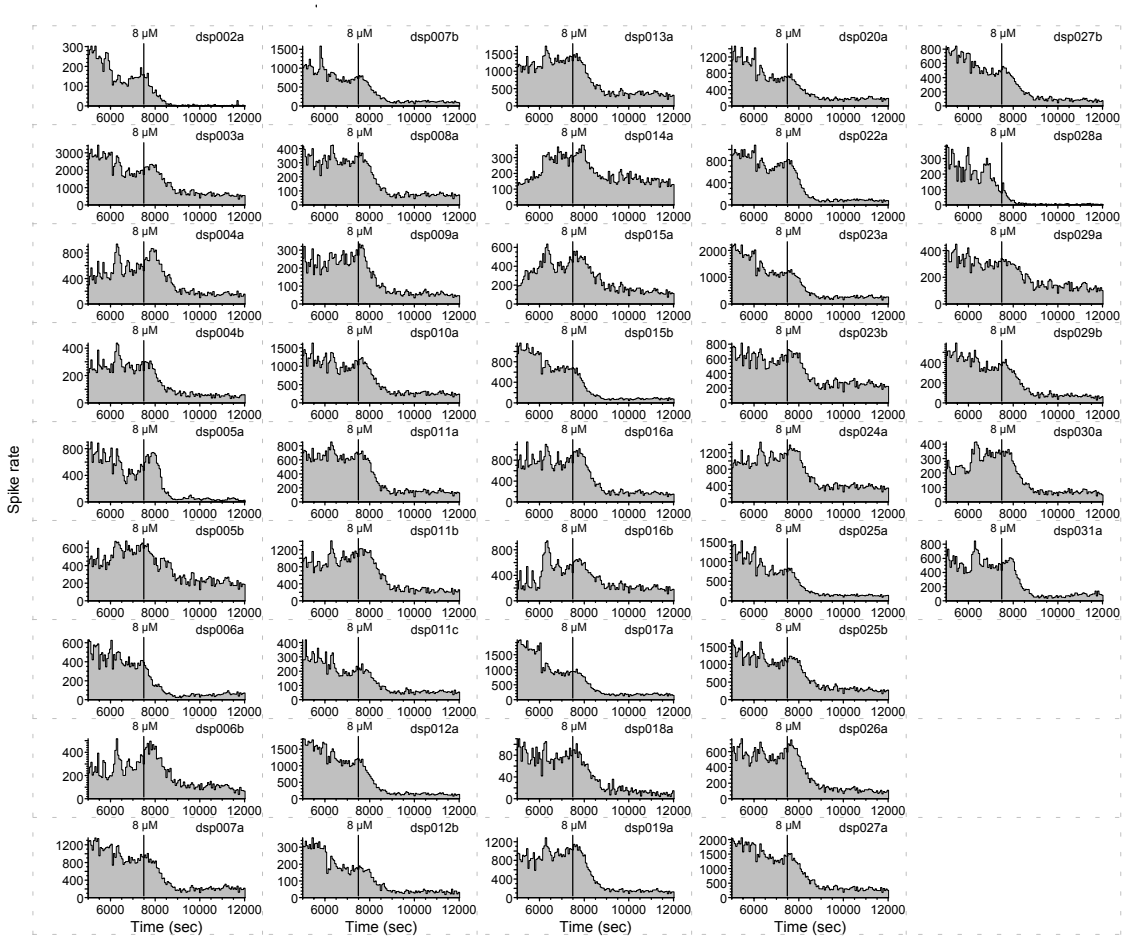


Figure 10. The uniform response of discriminated units to fluoxetine. The graph shows the evolution of spike rates (Y-axis) over time (X-axis). All of the 42 units had similar responses to 8 μM fluoxetine.

Cell toxicity is the inhibition of cell growth or deterioration of cells due to excessive concentrations of a particular agent. Abnormal morphology, including multi-nucleated giant cells, granular appearance, unusual vacuoles, and tattered cell edges, indicates cytotoxicity in morphology. Application of fluoxetine did not induce the above changes under microscopic observation.

Figure 11 displays the typical response of frontal cortex cultures when exposed to fluoxetine. The mean spikes/minute and mean bursts/minute averaged across the 30 channels were plotted against time. The reference activity of this frontal cortex culture was stabilized at mean spike rates of 1000/minute and mean burst rates of 27/minute. Fluoxetine at 0.5 μM did not produce obvious change in either spiking or bursting. At 6 μM, the network activity was suppressed to mean spike rates of 400/minute and burst rates of 12/minute. When concentration reached 13 μM, fluoxetine inhibited the spike rates and burst rates by 80% and 90%, respectively. Fluoxetine at 16 μM led to total stop of spiking and bursting.

Figure 12 shows a typical response followed by reversibility of frontal cortex cultures to low-concentration fluoxetine. At 3 μM, fluoxetine caused a 30% decrease in both spike rates and burst rates from the reference level. At 8 μM, fluoxetine blocked 75% spike rates and burst rates and 16 μM fluoxetine brought about cessation of activity. Two medium changes following exposure to 16 μM resulted in a return in

spike rates and burst rates, which was almost identical to the reference activity but showed less minute-to-minute fluctuation.

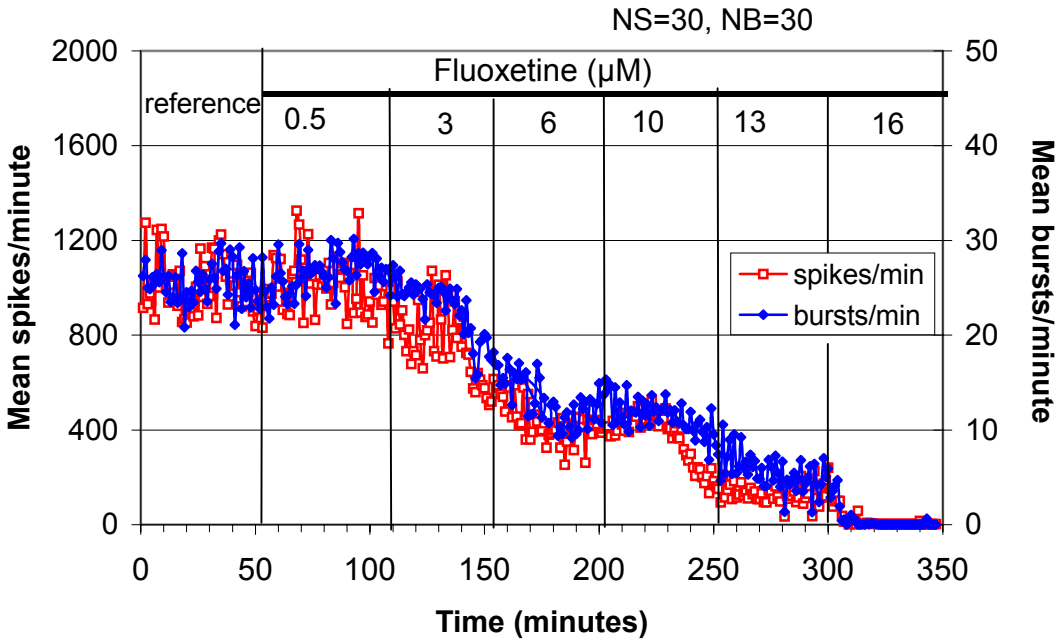


Figure 11. Typical response of frontal cortex culture to fluoxetine. At 0.5 μM, fluoxetine did not generate obvious effects. At 16 μM, fluoxetine completely stopped the activity. NS = number of units used for calculating mean spike rates; NB = number of units for calculating mean burst rates.

The inhibition caused by high concentrations of fluoxetine was partially recovered (Fig. 13). The network activity was shut off at 30 μM. After two medium changes, spiking and bursting were partially revived to 36% and 58% of reference, respectively. Neither of them returned to 100% of reference. Fluoxetine at 50 μM was applied to the culture and maintained for 90 minutes, which abolished all the activity. Two medium

changes led to partial recovery of activity. The spike rates and burst rates were restored to 37% and 60% of reference, respectively.

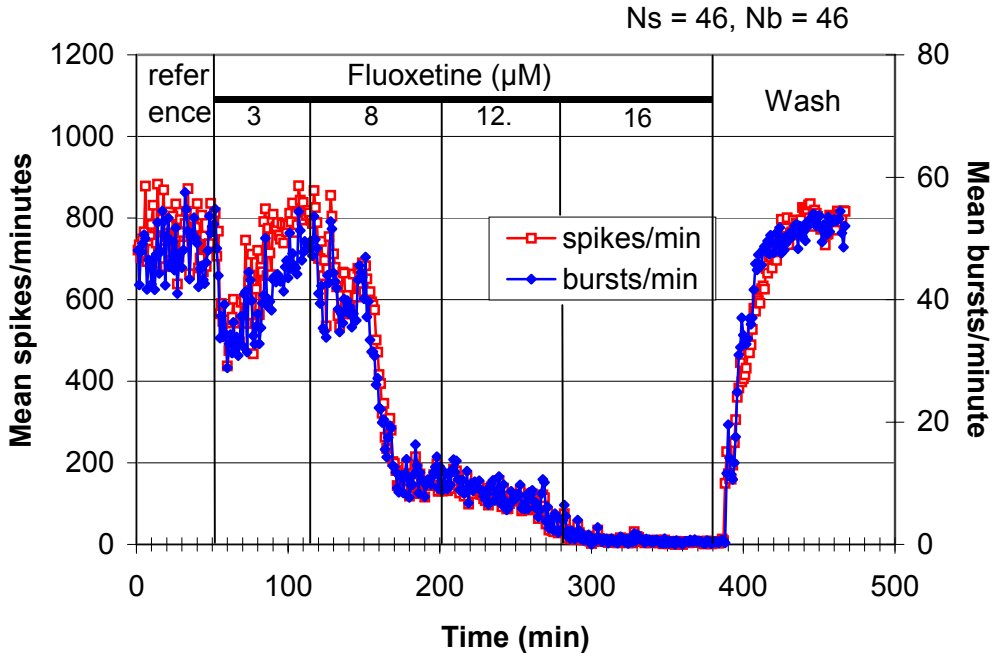


Figure 12. Response of frontal cortex cultures to fluoxetine with demonstration of reversibility. Initial decrease of activity occurred at 3 µM fluoxetine with activity being shut off at 16 µM. Two medium changes led to a return of spike rates and burst rates with less fluctuation compared to reference level.

Table 2 summarizes the 14 fluoxetine experiments. The percent change of spike and burst rates under certain fluoxetine concentrations were listed and the mean and S.D. of percent change were calculated. Dose-response curves of the 14 frontal cortex cultures based on this table are shown in Figure 14. Figure 14 (A) is dose-response summary

using mean spike rates ± S.D. Cessation of spike production occurred at 10-16 µM. EC50 is 4.1 ± 1.5 µM. Figure 14 (B) is the dose-response curve of fluoxetine using mean burst rates ± S.D. EC 50 is 4.5 ± 1.1 µM. Fluoxetine at 10-16 µM led to cessation of bursting.

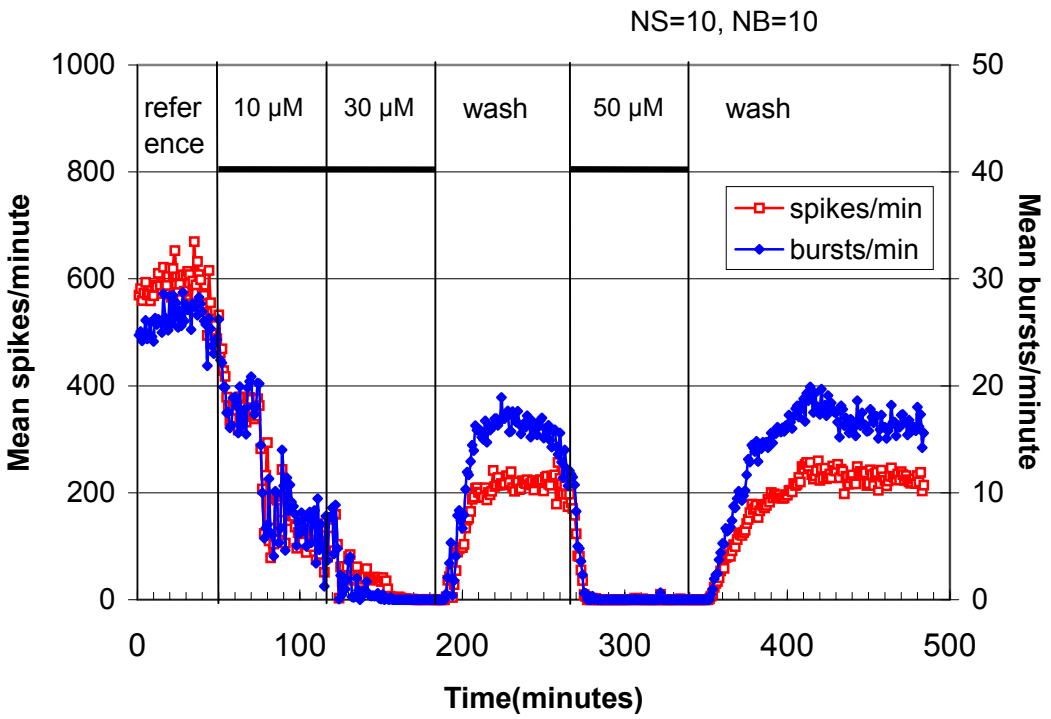


Figure 13. Fluoxetine suppressed spiking and bursting of frontal cortex cultures. The activity was shut off at 30 µM. Medium change following exposure to 30 µM and 50 µM fluoxetine led only to a partial recovery of activity.

Summary of Fluoxetine Experiments on Frontal Cortex Cultures

1. Spike Rate Change

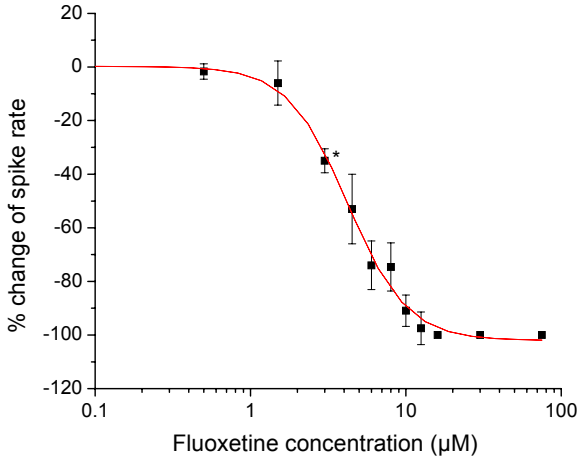
Exp	age	Fluoxetine Concentration (μM)													
No	(d.i.v)	0.5	1.5	3	4.5	6	8	10	12.5	16	17.5	20	25	30	75
		Percent change (%) of spike rate													
YX39	58			-35				-80							
YX43	28		0	-30	-65	-80		-90	-100						
YX44	28		-15	-40	-50		-55	-100							
YX45	44		-15	-40	-60	-70	-75	-90	-100	-100					
YX47	34		0			-85		-90							
YX48	54							-100					-100		
YX51	29	0	0				-75		-100						
YX53	21	0		-30		-65		-90	-100	-100					
YX55	28						-70	-90	-100	-100	-100				
YX56	46			-35			-78		-85	-100	-100				
YX57	47							-85		-100		-100			
YX58	31				-35			-95		-100		-100			
YX59	26							-90						-100	-100
YX60	30	-5													
Mean		-1.7	-6	-35	-53	-75	-71	-91	-98	-100	-100	-100	-100	-100	-100
SD		2.89	8.22	4.47	13.2	9.13	9.18	5.84	6.12	0	0	0	0	0	0

2 Burst Rate Change

Exp	age	Fluoxetine Concentration (μM)													
No	(d.i.v)	0.5	1.5	3	4.5	6	8	10	12.5	16	17.5	20	25	30	75
		Percent change (%) of burst rate													
YX39	58			-25				-80							
YX43	28		6	-20	-55	-75		-90	-100						
YX44	28		-10	-30	-50		-50	-100							
YX45	44		0	-20	-55	-65	-75	-90	-100	-100					
YX47	34		0			-80		-80							
YX48	54							-100					-100		
YX51	29	0	0				-65		-100						
YX53	21	0		-20		-65		-90	-100	-100					
YX55	28						-75	-90	-100	-100	-100				
YX56	46			-30			-75		-85	-100	-100				
YX57	47							-85		-100		-100			
YX58	31				-30			-85		-100		-100			
YX59	26							-90						-100	-100
YX60	30	-5													
Mean		-1.7	-0.8	-24	-48	-71	-68	-89	-98	-100	-100	-100	-100	-100	-100
SD		2.89	5.76	4.92	11.9	7.5	11	6.64	6.12	0	0	0	0	0	0

Table 2. Summary of fluoxetine effect on 14 frontal cortex cultures.

A.



B.

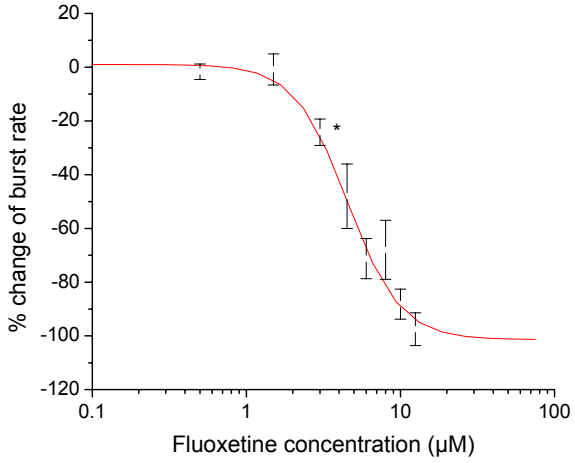
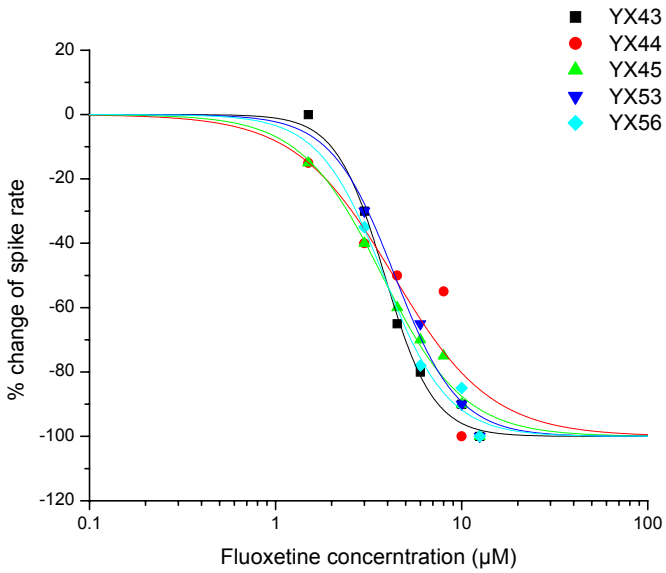


Figure 14. (A). Dose-response summary for fluoxetine using network spike rates. Cessation of spike production occurred at 10-16 μM. EC50 mean ± S.D. was 4.1 ± 1.5 μM. (B). Dose response summary of 14 frontal cortex cultures using burst rates. Cessation occurred at 10-16 μM. The EC50 was 4.5 ± 1.1 μM. * indicates the initial concentration that causes statistically significant change from control period, $P < 0.05$, one way ANOVA with post hoc Dunnett's test.

Figure 15 shows dose-response curves from five individual frontal cortex cultures. This is another way to calculate a population EC50. From the five individual curves, the resulting EC50 was 4.1 ± 0.3 µM. This method shows the inter-culture variability.



Exp No	EC50 (µM)
YX43	3.9
YX44	4.4
YX45	3.8
YX53	4.4
YX56	3.8
Mean	4.1
SD	0.3

Figure 15. The summary of dose-response curves from 5 individual cultures using spike rate data. The EC50 of each curve is shown in the table. With this analysis, EC50 mean ± S.D. was 4.1 ± 0.3 µM.

The dose-dependent inhibition of burst rates caused by fluoxetine was also associated with burst pattern changes. The integrated burst amplitude reflects spike frequency in the burst and increases with higher frequencies. It was observed that integrated burst amplitude decreased simultaneously with burst rates. However, burst amplitude changed at a slower rate compared to burst rates. The burst rates and burst amplitude relationship is shown in Figure 16. Native burst rates and burst amplitude

were used as reference (100%). Fluoxetine at 0.5 μM did not produce an obvious change in either burst rates or burst amplitude. Fluoxetine at 4.5 μM caused 53 ± 8% inhibition in burst rates with 28 ± 10% inhibition in burst amplitude. Fluoxetine at 12.5 μM led to 98 ± 6% change in burst rates. However, it led to only a 46 ± 8% change in burst amplitude (Fig. 16). This suggests that burst initiation is affected more by fluoxetine than the character of the burst, i.e., spikes and spike frequency in bursts, once the burst is ignited.

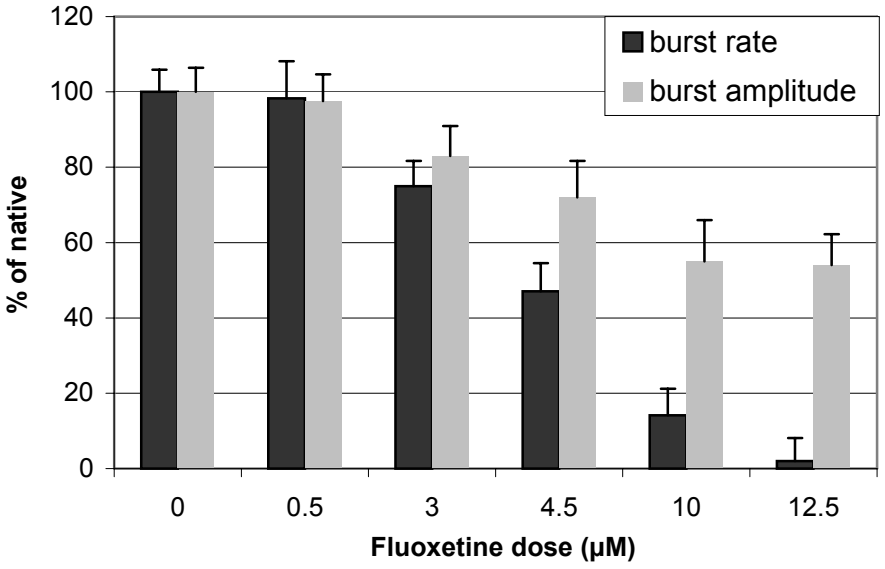


Figure 16. Summary of burst rates - burst amplitude relationship from 7 cultures. Burst rates had a more rapid change compared to burst amplitude (vertical bar represented mean ± S.D.).

The relationship between burst rates and burst duration in response to fluoxetine is displayed in Figure 17. When applied at 0.5 μM, fluoxetine did not produce significant change. At 3 μM, it caused 25 ± 6% decrease in burst rates and 58 ± 6% decrease in burst duration. Application of fluoxetine at 12.5 μM caused 96 ± 10% inhibitions in burst amplitude and 85 ± 13% decrease in burst duration.

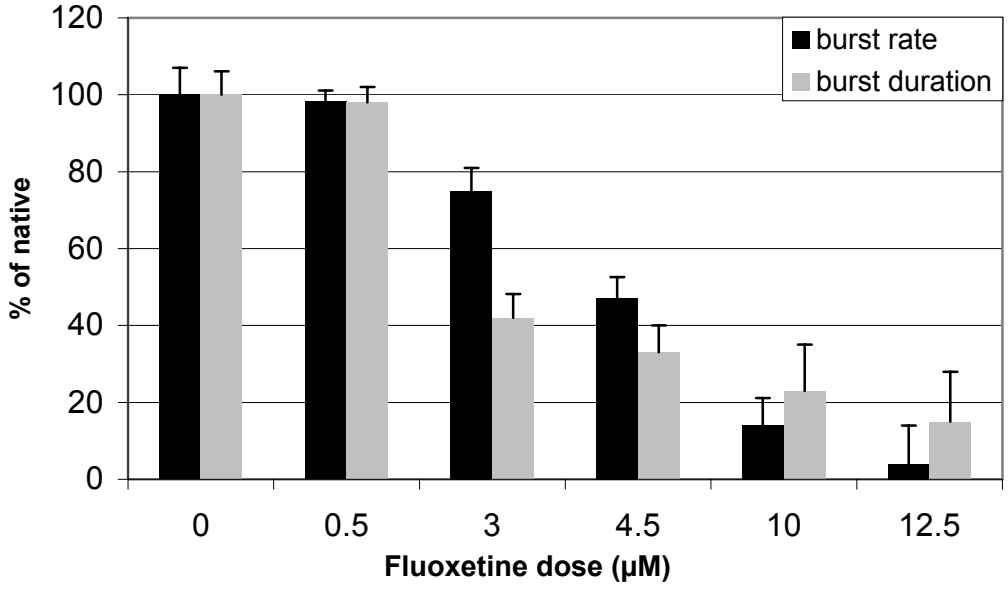


Figure 17. The relationship of burst rates and burst duration in response to fluoxetine. When applied at 0.5 μM, fluoxetine did not produce significant change. Application of 12.5 μM fluoxetine caused burst rates to decrease to 4 ± 10% of reference and burst duration to 15 ± 13%.

3.3 Responses of Cultured Frontal Cortex Networks to Ethanol

The control of ethanol concentration is difficult because ethanol is highly volatile. In addition, the cultures were under continuous CO_2 / air flow to maintain pH, which sped up the change of ethanol concentration in medium. To maintain ethanol concentrations, ethanol was put into the wash bottle and ethanol concentrations were monitored with a diagnostic kit. Figure 18 shows the results of ethanol concentration measurements. The ethanol concentrations in medium changed at a much slower rate when ethanol was added to the wash bottle.

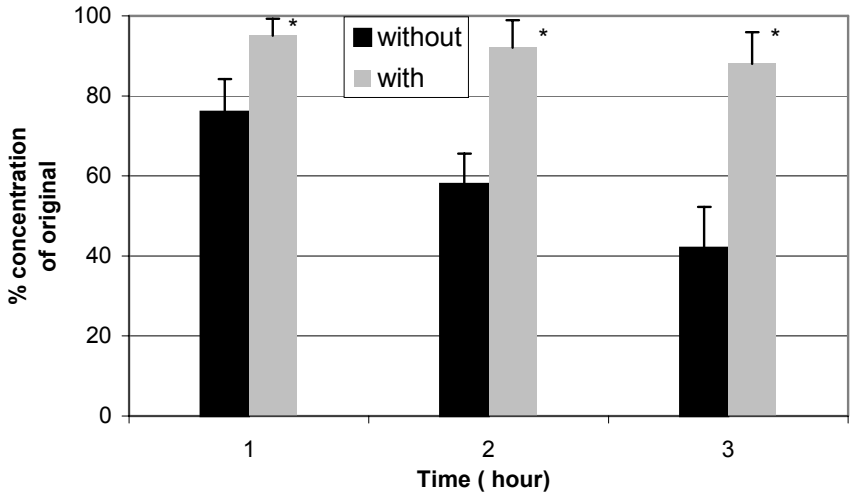


Figure 18. The measurements of ethanol concentrations in the culture medium. The data show percent change of concentrations against time without (black column) and with (gray column) ethanol in the wash bottle. Results represented mean ± S.D. from 6 separate experiments. * indicates statistically significant difference of the results without and with ethanol in the wash bottle after the same time periods (independent student's t test, $p < 0.05$)

Table 3 is a list of the 15 experiments included in the study of ethanol. The ages of the cultures ranged from 21 days to 61 days *in vitro*.

Experiment	Date	Age (d.i.v)	% Active channels	Max SNR	Avg SNR
Yx15	11/27/2000	30	25	3	2.5
YX16	12/18/2000	31	19	4	2.5
YX17	01/11/2001	21	20	6	3
YX18	01/18/2001	21	25	5	3.5
YX20	02/18/2001	46	24	5	3
YX21	02/26/2001	53	26	10	3.5
YX22	03/04/2001	61	32	5	3
YX25	03/31/2001	22	23	5	3
YX27	04/15/2001	40	33	5	2.5
YX28	04/22/2001	22	41	7	3
YX29	04/29/2001	30	22	3	2.5
YX30	06/03/2001	33	35	6	3.5
YX31	06/10/2001	50	22	8	3.5
YX34	6/31/2001	42	31	8	3.5
Mean		36	27	6	3

Table 3. List of frontal cortex cultures used in the study of ethanol. (d.i.v = days *in vitro*; SNR = signal-to-noise ratio)

Ethanol did not cause major change in action potential shape. Figure 19 depicts digitized action potential (waveform). Panel (A) shows the native waveforms of three units. Panel (B) shows the waveforms of the same units under 40 mM ethanol. No major waveform changes were found.

Ethanol inhibited the spiking and bursting in a dose-related way. Figure 20 is the rate histograms of 36 units from one culture. The spike rates of each unit were plotted against time. Among the 36 units, 40 mM ethanol decreased firing in 70%, increased firing in

16%, and generated no effect in 14%. The major effect was a reduction in activity. For all the ethanol experiments, 40 mM ethanol decreased firing in 71%, increased firing in 20%, and generated no effect in 9% (total unit numbers were 200). Consequently, unit-specific effects were observed.

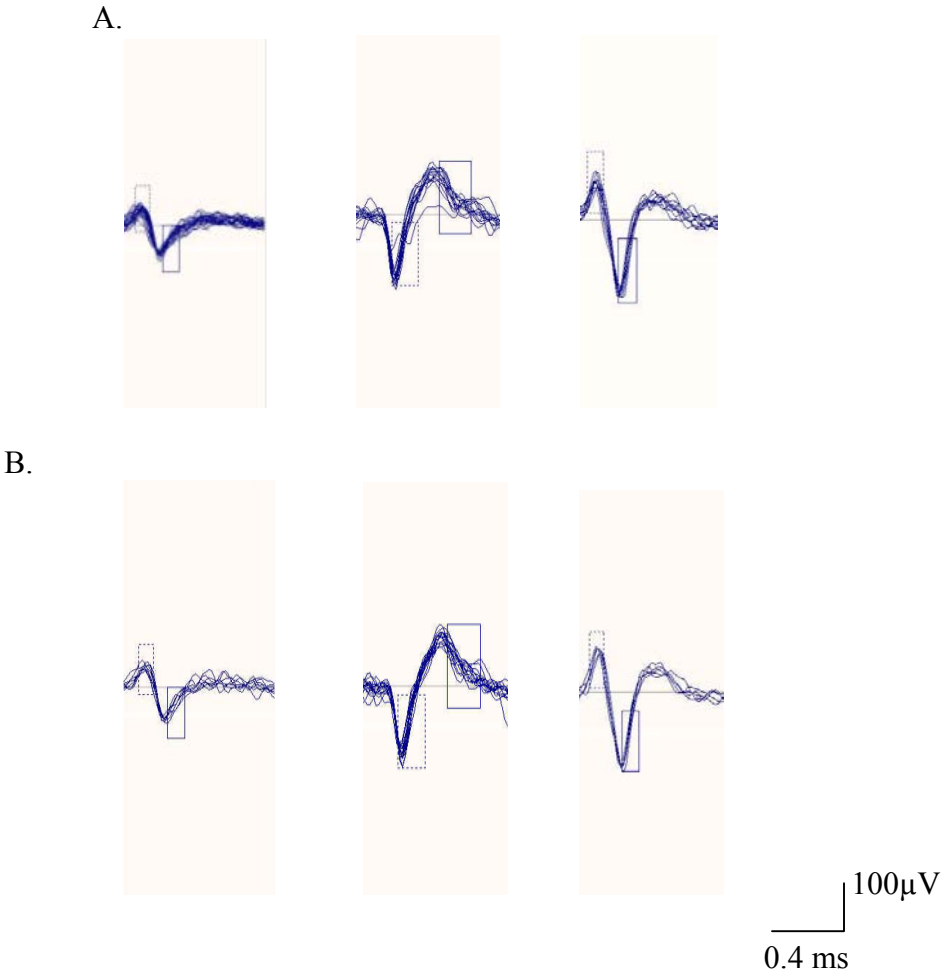


Figure 19. Panel (A) shows the native waveforms of three units. Panel (B) shows the waveform of the same units under exposure to 40 mM ethanol. The waveforms did not show major changes.

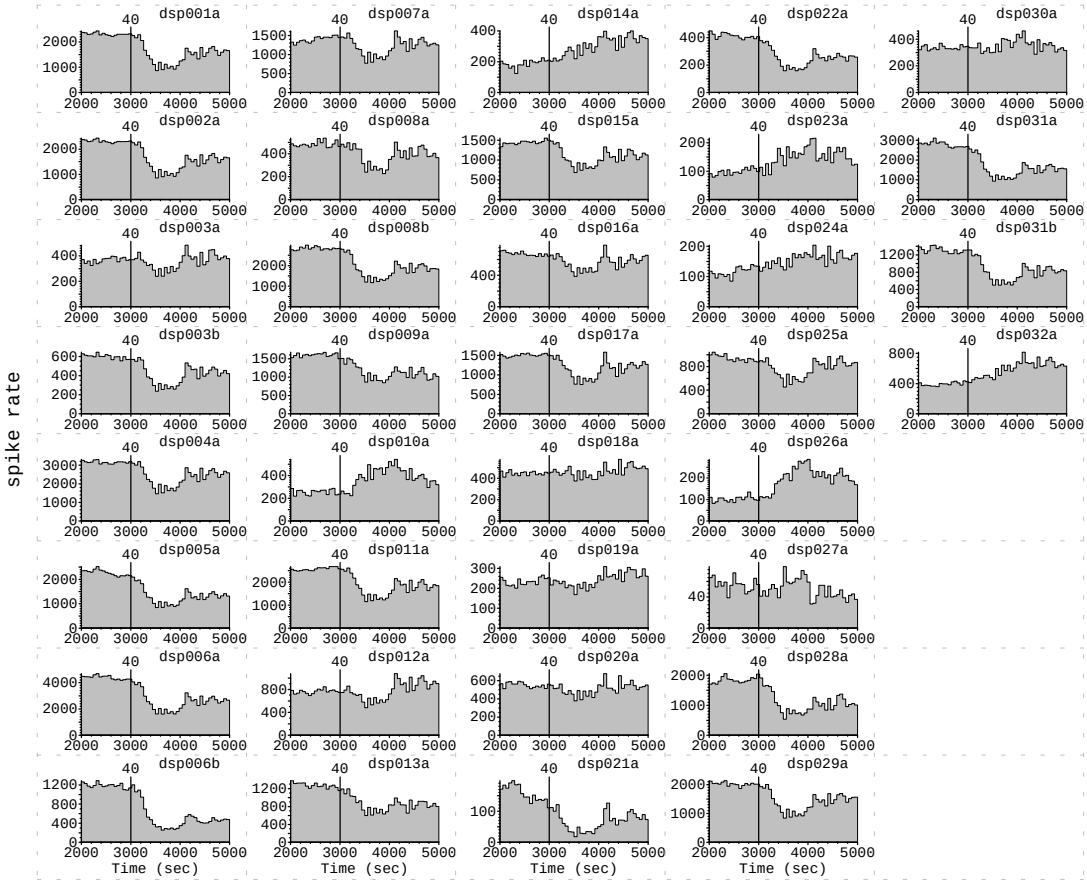


Figure 20. Responses to ethanol across the recorded units in one network. Among the 36 units, 40 mM ethanol (vertical line) decreased firing in 70%, increased firing in 16%, and generated no effect in 14%. The major effect was a reduction in activity.

Figure 21 shows typical activity change in response to ethanol. Activity decreased upon application of 20 mM ethanol. Progressive decrease in spiking and bursting occurred at increasing ethanol concentrations. Ethanol at 100 mM caused immediate network shut-off.

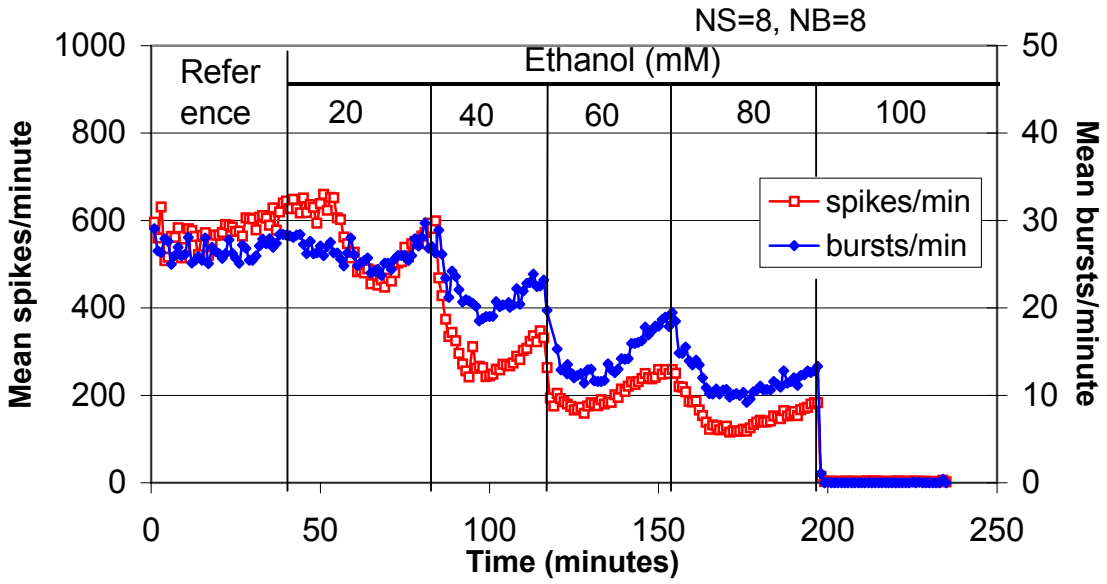


Figure 21. Effect of ethanol on a frontal cortex culture. A decrease occurred in spiking and bursting upon addition of 20 mM ethanol with progressive reduction with increasing concentrations. 100 mM ethanol shut off all the activity.

The inhibition caused by ethanol was reversible. Figure 22 shows that the activity returned following a medium change after exposure to 160 mM ethanol. Despite immediate 100% suppression of network activity, the network was restored to close-to-native spike and burst rates. Following the presence of 180 mM ethanol, only 25% spiking and 60% bursting could be revived.

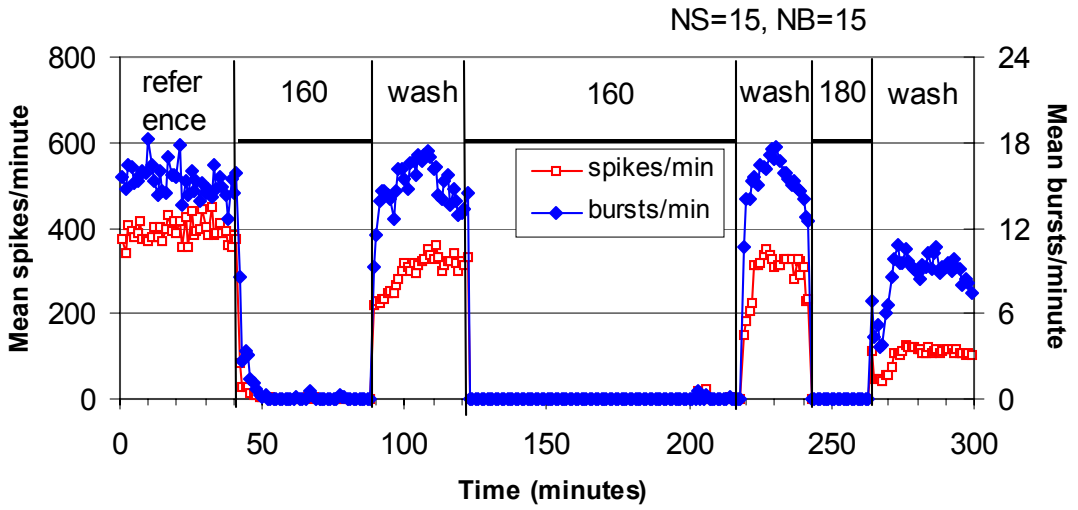


Figure 22. Reversibility of frontal cortex networks in response to ethanol. Ethanol at 160 mM caused total cessation of spiking and bursting. A complete medium change revived spike and burst rates to close-to-native state. After exposure to 180 mM ethanol for 20 minutes, a medium change caused only partial recovery of spiking and bursting to 25% and 60% of baseline, respectively.

Table 4 summarizes the responses of 15 frontal cortex cultures to ethanol. The percent change of spike rates and burst rates under the influence of ethanol was shown in this table.

Summary of Ethanol Experiments on Frontal Cortex Cultures

1. Spike Rate Change

Exp No	Age (d.i.v)	Concentration (mM) 10	20	40	60	80	100	120	140	180
		Percent change (%) of spike rate								
YX15	30				-40	-60	-90			
YX16	31	5	-30	-50	-55	-50				
YX17	20		-25	-60	-69	-83	-100			-100
YX18	20	-8	-15	-50	-53	-70		-100		
YX20	46			-60				-95		
YX21	53		-30	-40		-50	-85	-100		
YX22	61			-50	-65			-90		
YX25	22				-67	-60			-100	
YX26	30							-100		
YX27	40							-95	-100	
YX28	22								-100	-100
YX29	30					-80	-85	-90	-100	
YX30	33				-54			-90		-100
YX31	50						-100		-100	
YX34	42			-60						
Mean		-1.5	-25	-52.86	-57.57	-64.71	-92	-95	-100	-100
SD		9.19	7.07	7.53	10.2	13.38	7.58	4.63	0	0

2. Burst Rate Change

Exp No	Age (d.i.v)	Concentration (mM) 10	20	40	60	80	100	120	140	180
		Percent change (%) of burst rate								
YX15	30				-45	-58	-85			
YX16	31	0	-30	-60	-62	-65				
YX17	20		-10	-30	-48	-60	-100			-100
YX18	20	-10	-20	-45	-55	-70		-100		
YX20	46			-65				-90		
YX21	53		-10	-40		-55	-83	-100		
YX22	61			-50	-60			-90		
YX25	22				-63	-70			-100	
YX26	30							-100		
YX27	40							-95	-100	
YX28	22								-100	-100
YX29	30					-74	-90	-90	-100	
YX30	33				-65			-90		-100
YX31	50						-100		-100	
YX34	42			-70						
Mean		-5	-17.5	-48.33	-56.86	-57.45	-91.6	-94.38	-100	-100
SD		7.07	9.58	12.91	7.78	7.12	8.08	4.64	0	0

Table 4. Summary of 15 frontal cortex cultures in response to ethanol.

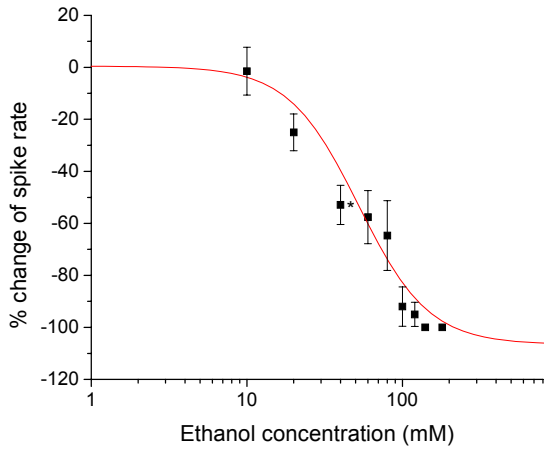
The ethanol dose-response curves using spike rates and burst rates based on table 4 are shown in Figure 23 (A) and (B). EC50s for spike and burst rates were 52.0 ± 17.4 mM and 56.0 ± 17.0 mM, respectively. In the presence of higher concentrations, the network activity was further decreased. 100-140 mM ethanol stopped both spiking and bursting in all networks.

Figure 24 shows the dose-response curves from four individual cultures using spike rates. The dose-response curves were obtained from each network separately. This is another way to get the EC50, which also shows the inter-culture variability. The resulting EC50 for spike rates is 48.8 ± 13.8 mM.

The relationship between burst rates and burst amplitude was important in identifying network response to certain compounds. Ethanol reduced burst amplitude by 10 ± 7%, 12 ± 12% and 15 ± 9% at concentrations of 40, 80 and 100 mM, respectively. In presence of the same concentrations, there was a greater dose-dependent inhibition on burst rates that averaged at 45 ± 9%, 57 ± 9%, and 83 ± 6%, respectively (Fig. 25).

The relationship between burst rates and burst duration is depicted in Figure 26. Ethanol led to concentration dependent inhibition of burst duration. Ethanol at 40 mM caused a 45 ± 9% decrease in burst rates and a 61 ± 9% decrease in burst duration. Ethanol at 100 mM caused 63 ± 9% decrease in burst duration.

A.



B.

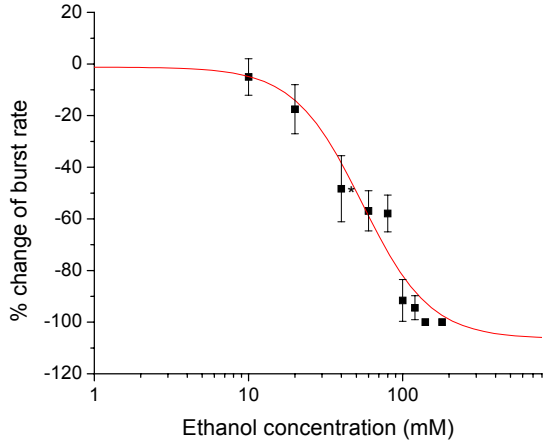
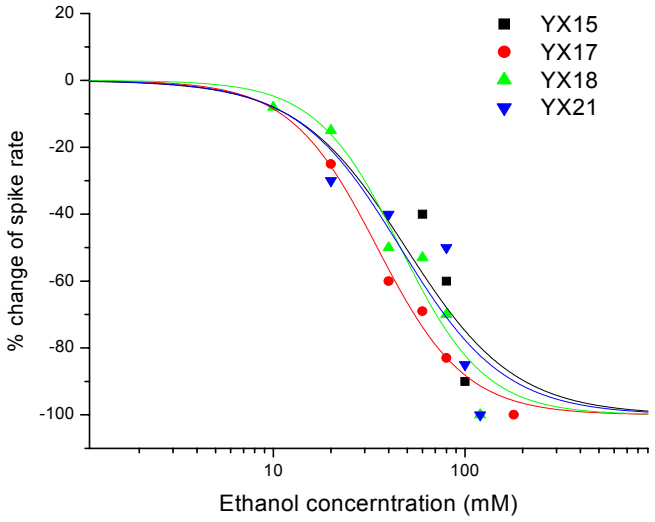


Figure 23. Dose-dependent response to ethanol using (A) spike rates and (B) burst rates. The curves summarized data from 15 experiments. Vertical bar represented mean and S.D. of activity change. EC50s were 52.0 ± 17.4 mM and 56.0 ± 17.0 mM for spike and burst rates, respectively. Concentrations above 100-140 mM led to cessation of spiking and bursting. * indicates the initial concentration that causes significant change from control period, $P < 0.05$, one way ANOVA with post hoc Dunnett's test.



Exp No	EC50(mM)
YX15	67.9
YX17	34.9
YX18	46.2
YX21	46.1
Mean	48.8
SD	13.8

Figure 24. The summary of dose-response curves of 4 frontal cortex cultures. EC50s of each curve are given in the table. This is another way to calculate EC50 mean ± S.D. and provide a better estimate of inter-culture variability.

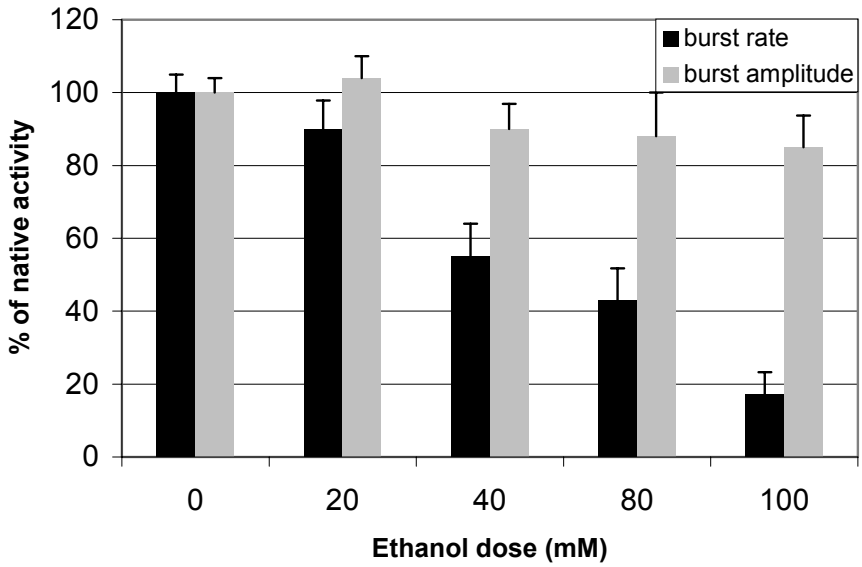


Figure 25. The relationship between burst rates and burst amplitude in response to increasing concentrations of ethanol. Ethanol caused a rapid change in burst rates. Raising ethanol caused burst amplitude to progressively decrease, but at a lower rate. Data were summarized from seven cultures.

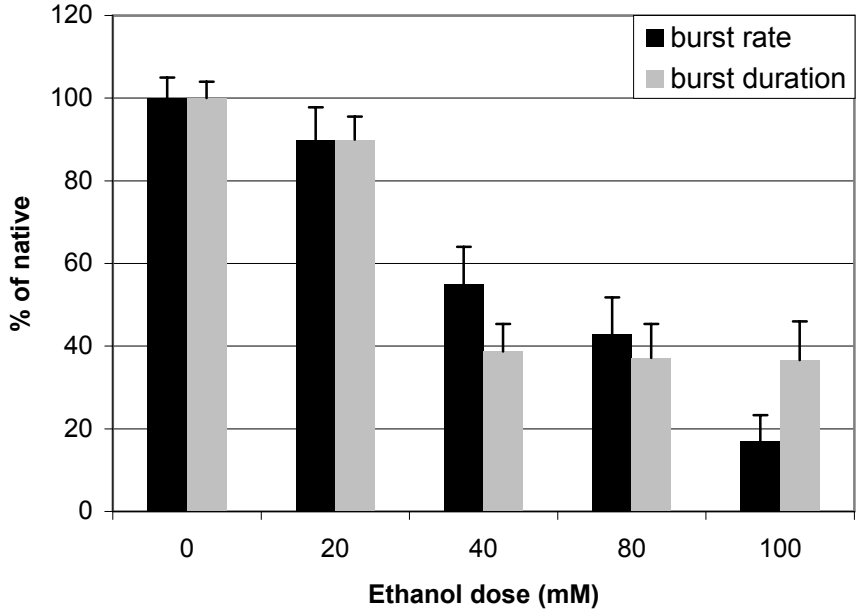


Figure 26. The relationship between burst rates and burst duration under exposure to ethanol. 40 mM ethanol caused a 45 ± 9% decrease in burst rates and a 61 ± 9% decrease in burst duration. Further decrease in burst duration was not observed before the total cessation of activity.

3.5 The Combination Effect of Fluoxetine and Ethanol

The combination effect of fluoxetine and ethanol on frontal cortex culture also was studied in this research. The ages of the cultures ranged from 30 to 45 days *in vitro*. Table 5 lists the experiments on the study of combination effects.

Neither amplitude nor shape of the action potential (waveform) was altered by co-application of fluoxetine and ethanol. Figure 27 displays the native waveforms (panel A) and the waveforms under the co-application of 5 μM fluoxetine and 20 mM ethanol (panel B) of three channels.

Exp No	Date	Age(d.i.v)	% active channels	Max SNR	Avg SNR
YX62	03/10/2002	30	43	9	3.5
YX63	03/16/2002	36	23	8	3.5
YX64	03/19/2002	45	30	7	4
YX65	03/23/2002	43	30	7	3

Table 5. Summary of the experiments on combination effect of fluoxetine and ethanol. d.i.v = days *in vitro*; SNR = signal-to-noise ratio.

Figure 28 displays the typical response of frontal cortex culture under exposure to fluoxetine and ethanol. The baseline spike and burst rates were 420/minute and 23/minute, respectively. Ethanol was maintained at 20 mM and fluoxetine concentrations were increased sequentially. Under 1 μM fluoxetine, the spiking decreased to 82% of baseline and bursting showed minor change. Under 5 μM fluoxetine, the spike rates decreased to 30% of baseline and burst rates dropped to 35% of baseline. When fluoxetine was increased to 10 μM, both spiking and bursting were stopped. Two medium changes partially revived the activity.

The combination application of fluoxetine and ethanol caused greater inhibition than the effect caused by fluoxetine or ethanol when applied alone. However, synergistic effects were not observed. Figure 29 is the dose response curves of fluoxetine + ethanol (dash curves) and fluoxetine only (solid curves). The combination application shifted the curves to the left, which implies an additive effect.

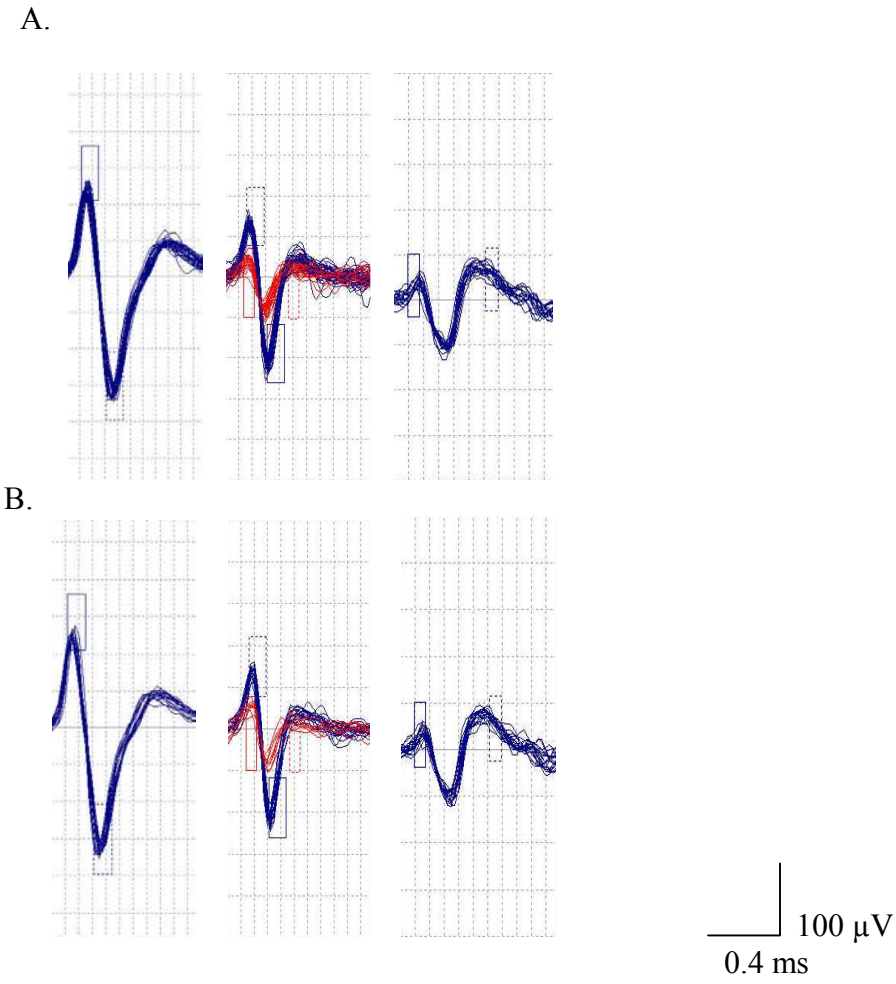


Figure 27. Panel (A) illustrates the native waveforms of three channels. Panel (B) exhibits the waveforms of the same channels when exposed to 5 µM fluoxetine and 20 mM ethanol. No major change in amplitude or shape was evident.

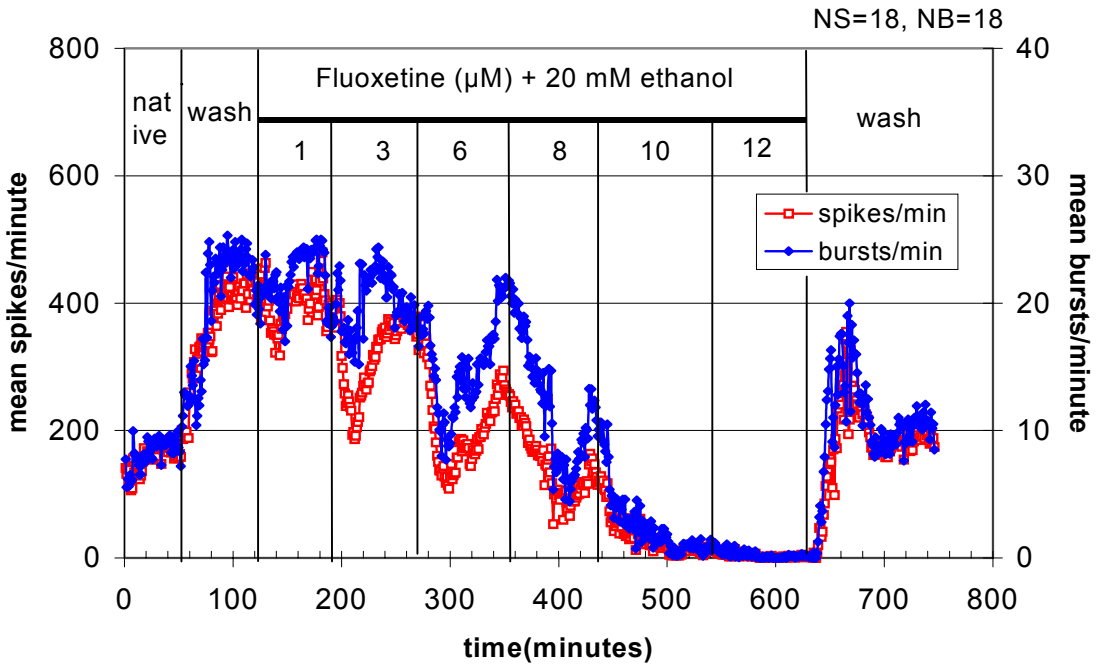
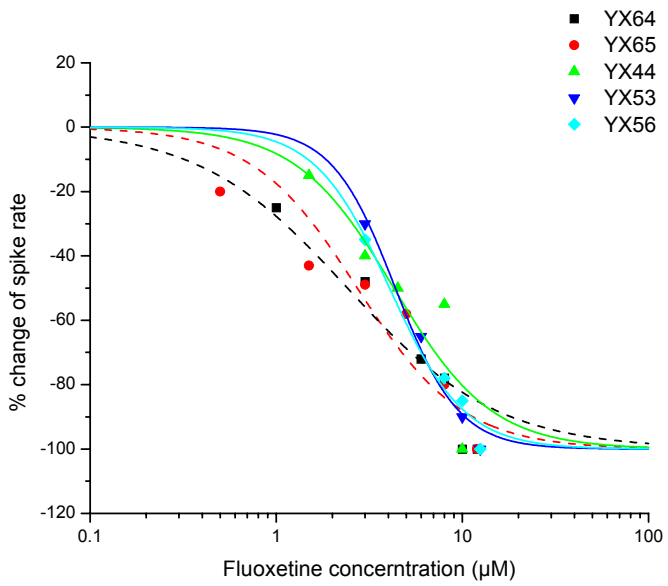


Figure 28. The combination effect of fluoxetine and ethanol on frontal cortex culture. Ethanol was maintained at 20 mM and fluoxetine concentrations were increased sequentially. Both spiking and bursting were stopped when fluoxetine was increased to 10 µM. Two medium changes partially revived the activity.



Exp No	EC50 (μM)	Exp No	EC50 (μM)
YX 64	2.7	YX 44	4.3
YX 65	2.4	YX 53	4.4
		YX 56	3.8
Mean	2.6	Mean	4.2
SD	0.2	SD	0.3

Figure 29. The comparison of dose-response curves of fluoxetine + 20 mM ethanol (dash curves, YX64 & YX65) and fluoxetine only (solid curves, YX 44,YX53 & YX56). The combination use shifts the curves to left and demonstrates greater inhibition than that caused by fluoxetine alone. The above table compares the EC50s from the experiments with application of fluoxetine plus 20 mM ethanol and fluoxetine alone.

CHAPTER 4

DISCUSSION

4.1 Effects of Fluoxetine on Frontal Cortex Cultures

Fluoxetine, a specific serotoinin reuptake inhibitor (SSRI), first appeared in the scientific literature in the 1974 issue of Life Sciences (Wong et al., 1974). It is referred to as a breakthrough in treating depression.

The serotonergic system is implicated in numerous behaviors and physiological functions, such as emotion, sleep, cognition and appetite. Decreased serotonergic neurotransmission plays an important role in the etiology of depression. The reuptake of serotonin into the pre-synaptic terminal via transporter is one pathway to terminate 5-HT action. Fluoxetine, with a high selectivity for 5-HT transporter, binds to 5-HT uptake sites in brain tissue (Wong et al., 1995) and thus modulates serotonin concentration in synapses.

There are more than 14 serotonin receptor types distributed over 7 distinctive receptors subfamilies (Andrade, 1998). Cortical neurons typically show a mixed inhibitory and excitatory response to 5-HT, which reflects the dual actions at 5-$HT_{2/1C}$ and 5-HT_{1A} receptors expressed by the same neurons. The direct action of 5-HT at the cellular level has a complicated effect. While 5-HT_{1A} agonist inhibits the serotonergic neurons in raphe nuclei, this inhibition may be the mixed effect of disinhibition for postsynaptic neurons that express 5-HT_{1A} receptors and disfacilitation for postsynaptic

neurons that express 5-HT_2, 5-HT_3, and 5-HT_4 receptors. Similarly, while 5-HT_2 agonist may directly excite a subpopulation of GABAergic interneurons in the cerebral cortex that express 5-HT_2 receptors, this excitation may be the mixed effect of inhibition for those pyramidal cells that receive input from these interneurons. Thus, functional consequence of the discrete cellular actions of 5-HT is closely related to the structure of neuronal networks (Aghajanian et al., 1990)

The clinical optimal dose range of fluoxetine is 20-40 mg/day and the therapeutic plasma concentration is 1 μM (Altamura et al., 1994). However, fluoxetine is lipid soluble with high brain-to-blood ratio ranging from 10 to 20 (Tsuneizumi et al., 1992; Karson et al., 1993) and the concentration in brain can be as high as 20 μM. However, fluoxetine is able to penetrate cell membranes and may distribute in intracellular components, so the concentration in synapses may be lower. An autoradiographic study with ^{18}F-fluoxetine showed that total binding in various brain areas such as frontal cortex, thalamus, hypothalamus, striata, cerebellum and olfactory tubercle was generally uniform (Mukherjee et al., 1998). The therapeutic brain concentration of 10 to 20 μM fluoxetine was close to the range used in my study. In my research, the effect of fluoxetine on the cultured cortical networks was tested, primarily to validate the neuronal network as a histiotypic system. When applied at 0.5 μM, fluoxetine did not produce obvious effects and the initially effective concentration was 3 μM. EC 50 for spike rates was 4.1 ± 1.5 μM and EC50 for burst rates was 4.5 ± 1.1 μM. Both spiking and bursting were abolished at 10-16 μM (Fig. 14 A and B).

Consistent with my observation that high concentration of fluoxetine arrested all the activity, it was reported that fluoxetine overdoses were fatal. One study reported that, among 633 adult patients who overdosed on fluoxetine hydrochloride alone, 34 resulted in a fatal outcome and 15 patients experienced different neurological and cardiovascular sequelea. The most common signs and symptoms associated with non-fatal overdosage were seizures, somnolence, nausea, tachycardia, and vomiting (website, http://www.rxlist.com/cgi/generic3/prozac_weekly_od.htm). Borys et al. (1992) reported 324 cases of fluoxetine overdose (232-1390 ng/mL, 0.67- 4.01 μM). Common symptoms included tachycardia, sedation, tremor, nausea, and emesis.

The elimination half-life of fluoxetine is 1-3 days after a single dose and 4 days after long-term administration. It is metabolized to the active metabolite norfluoxetine, which is also a specific serotonin reuptake inhibitor with an average elimination half-life of 7 days after long-term administration (Sommi et al., 1987). It is generally thought that a delay of 2-3 weeks is necessary before an antidepressant produces any improvement in the symptomatology of depressive patients. The delay is associated to the time needed to arrive at appropriate concentrations at target region and to regulate the monoaminergic system (down-regulation process). A study among 943 depressive patients, given 20 mg fluoxetine once a day, showed that the frequency distribution of the delay of an initial improvement mentioned by patients was bimodal with peaks at 5 and 11 days of treatment (Mesters et al., 1995). However, serotonin reuptake inhibitors rapidly block uptake sites or transporters, and cause neurochemical changes immediately. Thus, the

clinical efficacy cannot be entirely explained by direct blockade of 5-HT transporters. Other long-term changes should be taken into consideration (Hebert et al., 2001).

The data in this research showed that fluoxetine had an inhibitory effect on frontal cortex in the range of 3 to 16 μM. Along with the change in burst rates, the integrated burst amplitude and burst duration also showed dose-related diminution (Fig. 16 and Fig. 17). All these data suggested that fluoxetine may have anti-convulsant effects. It has been observed that enhancement of serotonin in brain exerts significant anticonvulsant effects on generalized epilepsy (Torre et al., 1970; Buterbaugh, 1978; Dailey et al., 1992; 1996; Prendiville and Gale, 1993). Reduced serotonin level showed proconvulsant effect (Kilian And Frey, 1973; Statnick et al., 1996). The anticonvulsant effects of serotonin reuptake inhibitors have been indicated in a variety of experimental models. Co-administration of fluoxetine and anticonvulsants enhanced the anticonvulsant effect in mice. A clinic study reported seizure occurrence after sudden stop of fluoxetine (Epelde et al., 1999). The antiseizure effect was found to be dependent on brain serotonin (Yan et al., 1994b; Pasini et al., 1996). Wada et al. (1999) found that concomitant administration of fluoxetine and 5-HT_{1A} receptor antagonist produced a significant increase in the threshold of focal hippocampal seizures in rats and thus decreased seizure. The inhibitory effect was abolished by pre-depleting brain 5-HT. Lu et al. (1998) documented that field excitatory postsynaptic potential and epileptiform activity were reversibly depressed by serotonin in the rat hippocampus. The studies supported that increased 5-HT neurotransmission in the hippocampus can inhibit seizures and the anticonvulsant action

of fluoxetine is due to increasing level of endogenous serotonin. Yan et al. (1994a) found that fluoxetine (intraperitoneal injection) diminished the audiogenic seizure intensity in genetically epilepsy-prone rats and proposed that a serotonergic mechanism was involved in the antiseizure effect of fluoxetine. Two exploratory experiments included in this research showed that application of 1-300 μM serotonin decreased both spike rates and burst rates (data not shown). However, it did not even reduce the network activity by 30%. The data suggested that the other mechanisms might be involved in the cessation of activity after application of fluoxetine.

Exposure to fluoxetine did not produce apparent change in waveform amplitude or shape (Fig. 9). Neural spike change was a reflection of alteration in membrane potential as a function of time. The consistency of waveform suggests that low-concentration fluoxetine does not affect the Na^+ or K^+ channels that are involved in action potential generation. However, it is interesting to note that fluoxetine shuts off all spiking in the network. Such a shut-off is not seen with botulinum toxin or tetanus toxin. This implies that fluoxetine may have secondary effects on spike generation at high concentrations.

In addition to its actions on the well-known action on serotonin reuptake system, fluoxetine has a variety of other effects. Garcia-Colunga et al. (1997) reported that neuronal nicotinic acetylcholine receptors were blocked by fluoxetine in a noncompetitive and voltage-dependent way. Nicotinic receptors are widely distributed in

the central nervous system, and thus the blocking action of fluoxetine on nicotinic receptors may play an important role in the antidepressant and other therapeutical effects.

4.2 Effects of Ethanol on Frontal Cortex Cultures

Among all the organ systems affected by ethanol, the central nervous system manifests the most obvious effect with immediate motor and emotional changes. The actions of ethanol are very complex and the mechanisms are not well understood.

The data in this study showed that acute exposure to ethanol decreased activity of frontal cortex cultures. The tested doses ranged from 10 mM to 180 mM. EC 50s were 52.0 ± 17.4 mM for spike rates and 56.0 ± 17.0 mM for burst rates. At concentrations as high as 100-140 mM, ethanol abolished both spiking and bursting (Fig. 23). Ethanol caused minor change in burst amplitude and major decrease in burst duration (Fig. 25 and 26). The action potential (waveform) did not change under the influence of ethanol (Fig. 19). It is interesting to note that wash after application of 180 mM ethanol caused only partial recovery. It is not certain if cell death has occurred and further studies may provide explanation on this issue.

This dose-dependent effect of ethanol was consistent with previous studies which addressed the association of blood concentrations of ethanol with certain behavioral effects. In mice, 40 mM led to a lack of righting reflex and 122 mM led to sleep and hyothermia (Little, 1990). In rats, 20 mM ethanol caused sedation. In human, 5-15 mM ethanol caused slight impaired attention, judgment and coordination, loss of efficiency in

fine performance, and minor euphoria. Ethanol at 15-30 mM resulted in significant sedation, ataxia, and confusion (Charness et al., 1989). Ethanol at 30-55 mM induced total mental confusion. Concentration at 50-100 mM led to loss of consciousness and central depression. Coma and respiratory arrest occurred when concentration reached above 100 mM. Ethanol consumption leads to change in psychomotor performance. It was reported that 0.1g/100dL BAL ethanol (22 mM) was associated with a 10% slowing of reaction time, increased errors and an increase in serious road accidents (Borkenstein, 1964; Robertson and Drummer, 1994). All these observations were made in the concentration range of 10-100 mM that was effective in cell cultures.

The effects of ethanol on neuronal firing were proposed in some studies. Benson et al. (1989) reported that 50-100 mM ethanol suppressed the firing of CA1 pyramidal neuronal in hippocampal slices. This effect was not attributed to changes of either membrane potential or membrane conductance. It was observed that ethanol (intraperitoneal injection, 1-4g/kg, blood concentration around 27-108 mM) caused a decrease in the spontaneous activity of Purkinje cells (Sorensen at al., 1981). Givens and Breese (1990) observed that ethanol (intraperitoneal injection, 0.75-3.0 g/kg, blood concentration around 20-80 mM) reduced neural firing of medial septal cells in a dose-dependent fashion and ethanol disrupted the rhythmic bursting pattern of medial septal neurons. Grupp (1980) observed that low concentration of ethanol caused an increase in the neuronal firing frequency or an excitatory effect followed by some reduction in firing

rates. Increasing concentrations produced augmented inhibition in firing. The changes in unit firing appeared to be correlated with the pattern of fronto-cortical EEG activity.

Some studies suggested that ethanol disrupts synaptic transmission in cerebral cortex and reduces or abolishes evoked patterns of activity. Chapin and Woodward (1983) reported that ethanol inhibited selective gating of somatosensory input to cortical neurons. Gruol (1982) found that, in cultured spinal cord neurons, ethanol caused a reduction in spontaneous activity at concentration as low as 20-30 mM. The rates of spontaneous EPSPs and IPSPs displayed major changes. However, the amplitude of these potentials remained the same. It was possible that ethanol influenced the firing of presynaptic neurons but not the synaptic transmission. The data in my research also supported that ethanol did not change the shape or amplitude of action potential before the total cessation of activity.

It was reported that acute exposure to ethanol caused enhanced $GABA_A$ receptor function (Chandler et al., 1998; Nishio and Narahashi, 1990; Reynolds and Prasad, 1991; Soldo et al., 1998; Tatebayashi et al., 1998). Lovinger et al. (1989;1990) studied the effect of ethanol on glutamate-activated channels in cultured murine hippocampal neurons with patch-clamp technique. Ethanol significantly decreased the amplitude of NMDA-activated current at the concentration ranges of 5-50 mM with IC50 at 30 mM. Application of ethanol at intoxicating concentrations on cultured murine cortex and spinal cord neurons showed similar effect. Increasing concentrations of ethanol caused increasing suppression of NMDA receptor-mediated EPSPs within concentration ranges

of 1-50 mM. These observations suggested that the inhibition of NMDA receptor/ionophore complex might contribute to acute ethanol intoxication. At low doses, ethanol produced euphoria and anxiolytic effect. The euphoric effect could be related to endogenous opiate and dopamine system.

There also have been a number of studies on the effect of ethanol on inhibitory transmitter-γ-aminobutyric acid (GABA), which generated different results. It is still uncertain whether ethanol potentiates GABA-mediated transmission or not. Procter et al. (1992) reported ethanol potentiated $GABA_A$ receptor-mediated EPSP in cerebral cortex. The effects of ethanol on the $GABA_A$-stimulated Cl^- current were potentiated in cultured mouse hippocampal, spinal and cortical neurons (Aguayo and Pancetti, 1994). Ethanol at 10-50 mM elicited a dose-related potentiation of GABA-mediated Cl^- flux in the cultured spinal cord neurons (Allan and Harris, 1986; Suzdak et al., 1988). However, some studies failed to see the potential effect of ethanol on GABA. It was reported that ethanol (10-200 mM) did not produce any effect on hippocampal neurons (Gage and Robertson, 1985).

4.3 Combination Effect of Fluoxetine and Ethanol

It is suggested that individuals taking fluoxetine should avoid ethanol during the course of treatment because ethanol may increase the sedative effects of fluoxetine. Although fluoxetine is generally thought to have a good safety in overdose, very high dose can cause serious side effects, such as seizures, electrocardiogram (ECG) changes,

and decreased consciousness. This toxicity is usually increased while taking ethanol simultaneously. Several fatalities due to combined use of fluoxetine and ethanol have been reported. Barbey and Roose (1998) reported that SSRI overdoses in combination with ethanol intake led to increased toxicity. Although it is cautioned that ethanol intake should be restricted during fluoxetine medication, only a very limited number of studies exist on the combination effect of the two compounds. The data in my research show that co-administration of fluoxetine and 20 mM ethanol shifted the dose-response curves to the left, which implies an additive effect (Fig. 29). Based on the ethanol data, 20 mM ethanol is not highly effective and reduced the network activity slightly. Consequently, the combination use of fluoxetine and ethanol produced more inhibitory effect. This research provided more evidence that ethanol should be restricted while on fluoxetine medication.

4.4 Application of Neuronal Networks

During drug development and pharmacological evaluation, an efficient and reliable experimental platform is needed before studies involve animal experiments. Neurons, with intrinsic electrophysiological mechanisms, can be utilized to detect ligand-receptor interactions, alteration in ion channels and membranes, and neurotoxicity. The cultured neuronal networks grown on microelectrode arrays show high sensitivity to the medium environment and provide rapid monitoring of this environment. The multi-unit recording of cultured neuronal networks is a valuable advance with many advantages, which

includes better control of compound concentrations, real-time monitoring of activity changes, and long-term experiments. Network mean spike and burst rates are the primary and simple parameters to study network activity. Many other features, such as integrated burst amplitude and burst duration, are also important in data analyses.

The cultures used in this research had different ages, seeding dates, cell densities, percentage of active channels and signal-to-noise ratios. The control of experimental environment, such as pH, osmolarity and temperature, is critical to reduce the intracultural and intercultural variability. However, small fluctuations are unavoidable and contribute to the variability of network responses. Errors resulting from drug mixing and concentration calculation also added to experimental variability. A higher stability of neuronal network could have been achieved if the experimental variables that may affect neuronal responses could be limited. Before improvement can be made, numerous studies have to be conducted to demonstrate that networks in culture are pharmacologically histiotypic. This research has contributed to this important effort.

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